Improved diagnostics to detect, describe, and understand pulpal and periapical inflammation

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Paper 3

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Paper 4

Rechenberg DK, Bostanci N, Zehnder M, Belibasakis GN. Periapical fluid RANKL and IL-8 are differentially regulated in pulpitis and apical periodontitis. *Cytokine.* 69: 116-119 (2014)

Paper 5

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A review article that summarizes the shortcomings of current pulpal and periapical diagnostic regimes, and envisages their potential improvement is presented as an introduction to the topic:

Rechenberg DK & Zehnder M. Molecular diagnostics in endodontics.
Endod Top 30: 51-65 (2014)

Background and Aim

Dental caries is one of the most prevalent infectious diseases worldwide, affecting the majority of children and adults. It represents an opportunistic infection of dental hard tissues by oral or transient microorganisms[1]. With the spread of the caries towards the dentin the pulp becomes inflamed, i.e. it develops a pulpitis[2]. If the caries lesion progresses further, microorganisms will gain direct access to the pulp space eventually. This results in local micro-abscess formation, digestion of the pulp tissue by proteolytic enzymes, and finally necrosis of the whole dental pulp[3]. As the infection spreads from the coronal to the apical aspects of the root canal system, the periapical tissues get involved. Microbial by-products and endotoxins cause inflammation and resorption of the periapical bone. Collectively, all the inflammatory stages of the periapex that are caused by the infection of the dental pulp space are called apical periodontits[4]. Hence, pulpitis and apical periodontitis are different, dynamically evolving inflammatory stages of endodontic microbial infection.

Medical diagnostic tests are performed to identify the presence of a specific disease and to determine its treatment. Histologic processing represents the gold standard for detecting pulpal and periapical inflammation[5]. For obvious reasons this is not applicable for teeth that are to be preserved. Therefore, current endodontic diagnostic procedures that aim to assess the stages of pulpal and periapical inflammation involve case history, as well as clinical and radiographic examination. The clinical examination includes different procedures such as inspection, pulp sensitivity to thermal or electric stimuli, and the assessment of pain on palpation or percussion. Based on these findings pulpal (normal, reversibly inflamed, irreversibly inflamed, or necrotic pulp) and periapical (normal apical tissues, apical periodontits, apical abscess, or condensing osteitis) diagnoses can be made[6].

The clinical procedures for pulpal diagnostics have not changed much in the last century[7]. However, a poor correlation between those tests and the actual histopathological state of the pulp has been revealed[5, 8]. A recently performed systematic review concluded that there is insufficient evidence for the accuracy of clinical endodontic tests in detecting the inflammatory state of the pulp, even if these tests are combined[9]. The authors concluded that current endodontic diagnostic procedures need to be improved, and that biological markers for pulpal inflammation warrant further investigation.

Periapical diagnostics essentially comprises the interpretation of periapical radiographs. However, the limitations of two-dimensional imaging in depicting periapical bone changes are well known. Apical periodontitis in cancellous bone, for example, radiographically can be covered by thick cortical bone. Consequently, apical inflammation can be missed even though extensive disease is present[10, 11]. Moreover, the apparent healing of apical periodontits on periapical radiographs can take up to 4 years, or even longer[12]. Theoretically these shortcomings may be overcome by 3-D imagining techniques

such as cone beam computed tomography (CBCT)[13]. However, because of the comparatively high radiation dose to oral tissues, CBCT is currently not recommended for standard periapical diagnostics, or for monitoring periapical health[14].

Since the current clinical endodontic diagnostic procedures are not necessarily reliable, the thus-resulting pulpal and periapical diagnoses are relatively poor. On the one hand, this can lead to over-treatment (i.e. pulpectomy in cases that could be kept vital), whereas on the other, treatment failures may remain undetected. Keeping in mind that the underlying cause for pulpal and periapical infections are microorganisms that cause inflammation by the host, endodontic diagnostics could also focus on either the extent of the microbial infection, or the inflammatory reaction of host tissues. Therefore, the aim of this cumulative synthesis of experiments is to refine current endodontic diagnostics and to investigate alternative diagnostic approaches, which target the presence of microorganisms, or the host response.

METHODS

Firstly, a prospective, observational study was performed enrolling emergency patients that visited the University of Zurich, Center of Dental Medicine because of acute pain caused by an endodontic infection. The patients were comprehensively examined and asked 11 specific questions from a checklist with a possible diagnostic discerning value between two forms of endodontic infections: acute apical periodontitis and acute (irreversible) pulpitis. In addition, the pain levels were assessed using a numerical rating scale (NRS-11). Subsequently, a decision tree was constructed based on recursive partitioning to identify a hierarchy in the symptoms differentiating between the two conditions.

Secondly, the currently available scientific literature was systematically analyzed in order to identify potential biological markers that may be used to target pulpal inflammation. An extensive electronic literature search was performed to look for studies that differentiated between a healthy and an irreversibly inflamed pulp in permanent human teeth by analyzing interstitial/ dentinal fluid, gingival crevicular fluid, pulpal tissue, periapical fluid or apical blood for the presence of a biological marker. The last date entered was February 19, 2015 and no language restrictions were imposed. The quantitative data was collected from all studies included to the review and the quality of theses studies was assessed. One promising substrate for molecular analysis was found to be dentinal fluid. However, the vehicle (a polyvinylidene fluoride membrane, PVDF) used for collecting the dentinal fluid in a pilot study was not perfectly suitable for this purpose[15].

Therefore, in a third study performed, alternative vehicles were compared to a PVDF counterpart for their ability to absorb water and release protein. In a subsequent comparative clinical trial, the cellulose vehicle with the most favorable outcome during pre-assessment

was compared to PVDF regarding the levels of matrix metalloproteinase-2 (MMP-2) that could be collected from dentinal fluid of healthy human teeth during filling replacement. The MMP-2 levels were determined by enzyme-linked immunosorbent assay (ELISA). Data from the laboratory experiments were compared between materials using appropriate parametric tests.

A further (fourth) attempt aimed at investigating the expression of host-factors that may serve as biological markers for periapical inflammation kinetics during the development of apical periodontitis. In a clinical trial, symptomatic teeth without the presence of apical periodontitis (diagnosed with irreversible pulpitis) were compared to teeth with symptomatic apical periodontitis. Cellulose paper points were used to collect periapical tissue fluid during root canal treatment of these teeth. The samples were evaluated for the presence of interleukin-8 (IL-8), receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG) using ELISA. Target protein levels per case were calibrated against the corresponding total protein content and statistically compared.

A fifth study and first of its kind focused on detecting key microbial taxa (*Synergistetes, Actinomyces, and Fusobacteria)* directly in periapical tissue fluid samples as described above in three defined stages of periapical disease: irreversible pulpitis but normal apical tissues, pulp necrotic teeth with apical periodontitis, or apical periodontitis associated with a root-filled tooth. Samples were investigated using FISH staining and epifluorescence microscopy. A semi-quantitative evaluation of the stained bacterial taxa was performed.

FINDINGS

The most accurate indicator for discerning between acute forms of pulpitis and apical periodontitis was absence of pain to cold stimuli in the latter condition. In eighty percent of the teeth with acute apical periodontitis patients did not report pain on cold. In teeth that did have a history of pain triggered by cold stimuli, the decision tree still correctly identified acute apical periodontitis in 72 % of the teeth that felt too high and had hurt for less than one week. The median pain levels reported by the patients diagnosed with acute forms of pulpitis and apical periodontitis did not show statistical differences ($P < 0.05$; median NRS-11 = 8, for both diagnoses). Consequently, pain levels are not useful to diagnostically differentiate between those conditions

From the 851 articles screened for the systematic review, 57 met the inclusion criteria and analyzed a potential biomarker to differentiate between a healthy and an irreversibly inflamed pulp. Most of the included studies evaluated pulp tissue (50/57), whereas merely 12% (7/57) analyzed other substrates such as pulpal blood (N = 2), or dentinal fluid (N = 1). From the 89 potential biological markers assessed in pulp tissue and the 16 evaluated in

other substrates, 71.9% and 75% respectively, revealed statistical significant differences between an irreversible inflamed and a healthy pulp.

A large-pore cellulose membrane was shown to be ideal for collecting biological markers from dentinal fluid. Compared to a standard PVDF membrane, the cellulose membrane absorbed significantly more water and released proteins as well as the PVDF membrane ($P < 0.05$). Matrix metalloproteinase-2 could be collected at an ELISA quantifiable level from the dentine of healthy teeth using the cellulose membrane in 9 of 13 cases, compared to merely 1 of 13 with the PVDF membrane ($P < 0.05$). Consequently, using large-pore cellulose should improve the analysis of low-available body fluids such as dentinal fluid, or periapical tissue fluid.

The periapical tissue fluid, which was collected from symptomatic teeth without the presence of apical periodontitis (irreversible pulpitis) and teeth with symptomatic apical periodontitis employing cellulose paper points, revealed a significantly ($P < 0.05$) negative correlation between RANKL and IL-8. High levels of RANKL, a molecular marker stimulating osteoclast activation and bone resorption, were present during initial periapical lesion development (i.e. during irreversible pulpitis). A high level of IL-8, a cytokine strongly associated with neutrophil recruitment and activation (i.e. with inflammation), was diagnostically indicative for already established periapical lesions. This suggests that in the development of apical periodontitis molecular activation of bone resorption takes place before inflammatory cell recruitment. However, OPG was under the detection limit in most samples regardless of their diagnosis. Apparently OPG does not bear much of diagnostic information for monitoring the periapical inflammatory reaction.

For the first time it was shown, that FISH can be used to detect individual taxa and even biofilm fragments in tissue fluid samples of teeth with different clinical conditions. It was shown that *Actinomyces* were significantly more prevalent in root-filled teeth with apical periodontitis (10/21) compared to counterparts with primary apical periodontitis (4/33) or pulpitis (0/27).

DISCUSSION

Collectively, the studies presented here address the diagnostics of endodontic disease. The current clinical diagnostic procedures for pulpal and periapical inflammation and infection indubitably have their shortcomings[5, 8]. However, it must be noted that the reliability of these tests greatly depends on the condition to be diagnosed. Their main weakness lies in discriminating a healthy condition from the beginning of the disease. In contrast, definite biological states can be diagnosed with good reliability. For example, it is possible to identify an irreversibly inflamed pulp in an acute painful tooth with high sensitivity[16]. And it could be shown that a tooth that does not react on cold testing and shows

a periapical translucency radiographically almost invariably contains a necrotic pulp[3]. Consequently, the diagnoses that were used here for the clinical trials can be regarded as sound. Rigorously combining and analyzing all potentially relevant information together in a checklist approach enabled constructing a hierarchy in differentiating symptoms for different conditions. Such information bears diagnostic value for performing cross-sectional studies and to diagnose and advice patients during out-of hours calls. However, unfortunately the current clinical diagnostic tests do not address all relevant questions to be asked. In the context of vital pulp treatment for example, the challenge lies in determining how far the inflammation of the pulp has actually progressed before it reaches an irreversibly inflamed condition[17]. The systematic review showed that there is a different expression of immunological factors in a healthy and an inflamed pulp on the molecular level. These markers may be used to determine (and diagnose) pulpal inflammation. However, the review also revealed that there is currently not much research available specifically designed for that purpose. Conversely, a group of experts in the field have demanded methods that target the extent of the microbial infection, or the inflammatory reaction of the host tissue[9]. Dentinal fluid analysis, as one example, could be applied to address the clinically relevant question of whether or not a pulp has a chance to survive a treatment such as direct capping, or an abutment preparation for placement of a crown[18, 19]. Studies, that are specifically designed to improve methodological issues, such as the sampling vehicle, build the foundation for their potential clinical application. The same applies for studies involving periapical tissue fluid analyzes. There are clearly advantages analyzing periapical tissue fluid for microbial infection and inflammatory host-factors. For example, a distinct periapical translucency on a radiograph represents a quantifiable endpoint for the presence of inflammation, and the pathology is accessible in an enclosed, circumscribed area[20, 21]. Here, it was possible to identify different endodontic key taxa in the periapical tissue fluid of teeth that differed with respect to their root canal content (root-filled vs. necrotic), but periapically showed the exact same diagnoses (chronic apical periodontitis) based on conventional diagnostic methods. Regarding the analysis of periapical tissue fluid for immunological host-factors to monitor periapical health, it would be of great interest to know as early as possible whether an apical periodontitis begins to heal after initiation of a root canal treatment. This was shown to be measurable by analyzing molecular markers in periapcial tissue fluid[22]. Knowing the dynamics, when molecular biological markers are expressed during the development of apical periodontitis (as investigated here), sets the framework for such applications.

Collectively, the experiments performed here point into the direction that such diagnostic methods based on molecular tests are applicable under well-controlled conditions and can be developed. However, many possible impasses have yet to be overcome before a

clinical application can be considered. It appears to be worth performing further research into this direction.

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Paper 1

Rechenberg DK, Held U, Burgstaller JM, Bosch G, Attin T.

Pain levels and typical symptoms of acute endodontic infections: a prospective, observational study.

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Pain levels and typical symptoms of acute endodontic infections: a prospective, observational study

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Abstract

Background: This study aimed to identify key symptoms that could be associated with the diagnosis of acute forms of symptomatic apical periodontitis (SAP) and symptomatic irreversible pulpitis (SIP), and to identify a diagnostic algorithm based on these symptoms.

Methods: In this prospective, observational study 173 emergency patients diagnosed with acute pain of endodontic origin and no swelling or fistula were included. Patients were asked 11 specific questions from a checklist with a possible discerning value between acute SAP and acute SIP. Pain levels were recorded using the numeric rating scale (NRS-11). Subsequently, the painful tooth was diagnosed. Logistic regression was used to evaluate the checklist regarding its differentiation between SAP ($N = 103$) and SIP ($N = 70$). Moreover, a decision tree was constructed based on recursive partitioning to identify a hierarchy in differentiating symptoms.

Results: With identical median NRS-11 scores of 8, the teeth diagnosed with acute SAP and SIP were severely painful. The decision tree analysis resulted in a tree with splits according to pain on cold, perceived tooth extrusion, and pain duration. The overall sensitivity of the tree to detect SAP based on key symptoms was 95 %, its specificity was 31 %.

Conclusions: The best indicator for SAP was a reported absence of pain to cold stimuli. In teeth that did have a history of pain triggered by cold stimuli, the decision tree correctly identified SAP in 72 % of the teeth that felt too high and had hurt for less than one week.

Keywords: Pain, Root canal, Symptom, Diagnostic

Background

Odontalgia is the main cause of oro-facial pain [1]. Acute dental pain is mostly attributed to microbial infection of the dentin being in close proximity to the pulp, the pulp space and finally the periapical tissues [2, 3]. The pulp, the periodontal ligament, and the periapical tissues form natural barriers, which help the host orchestrate a defense against invading opportunistic pathogens [4]. When these barriers are invaded with pathogens nociceptors are activated due to inflammation and tissue breakdown [5]. Three symptomatic clinical conditions deriving from endodontically involved teeth have been identified: symptomatic irreversible pulpitis (SIP), symptomatic apical periodontitis (SAP) and acute apical abscess (AAA) [6]. Even though these can be extremely painful [2, 7], they do not need to be [8, 9]. Unfortunately, the current diagnostic nomenclature of the American Association of Endodontists (AAE; [6]) does not differentiate between teeth that cause significant enough pain to require the patient to seek emergency care from those, which merely show an increased reaction to diagnostic tests [10].

The socioeconomic importance of dental pain has long been recognized [11]. It has thus been attempted to develop specific dental pain questionnaires for epidemiologic studies [12]. These questionnaires appear to have good predictive values to differentiate between groups of conditions, such as those that are caused by endodontic

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infections and those that are not. However, they do not differentiate the main causes of severe dental pain emanating from endodontic origin. There have been other approaches, which were more specific. However, these frequently mixed symptoms reported by the patients with clinical observations by the investigators [13, 14]. The significance of establishing the correct diagnosis of endodontic infections should not be under-estimated [15]. If endodontic infections are not treated appropriately, life-threatening conditions can evolve [16]. Due to the obvious clinical symptom of edema (swelling) associated with the diagnosis of AAA, this diagnosis poses no challenge. In contrast, clear-cut symptoms have not been identified to differentiate between SAP and SIP. While SIP is merely painful, SAP is the beginning of the spread of infection with a possibility of untoward systemic consequences [16]. Abscess formation, where bacteria are invariably present in the periodical tissues, marks one possible endpoint of this infection process [17]. Depending on the severity of the infection and the location of the tooth, SAP can even lead to the death of the patient if not treated appropriately [18]. Furthermore, the emergency treatment for SIP and SAP differs [15]. With SIP, simply removing the coronal pulp is sufficient for relief [19], while with SAP the disinfection of the entire root canal system is needed. It would thus be helpful to further investigate symptoms including pain levels and pain duration that can be specifically related to the acute forms of SIP and SAP.

In this prospective, observational study, adult patients seeking emergency care in a dental hospital because of inflammatory conditions caused by infection of the pulp space were assessed. The aim of the study was to identify key symptoms that could be associated with either SIP or SAP, and to identify a diagnostic algorithm based on these symptoms. Symptoms were related to clinical signs/findings.

Methods

Cohort identification and inclusion criteria

All patients attending the dental emergency unit at our institution from opening at 07:30 am to 10 am were considered. It was aimed to include all adult (18 years or older) patients presenting with acute pain from a permanent tooth caused by an endodontic infection. The emergency unit consisted of physicians and dentists from all dental specialties. After a short first examination by an oral surgeon, patients diagnosed with pericoronitis or temporomandibular joint pain were referred to the Oral Surgery department. All other patients were referred to the Department of Preventive Dentistry, Periodontology and Cariology for further examination. Patients who did not present with spontaneous pain, but merely reported slight discomfort to stimuli indicative of reversible pulpitis were not considered as'acute'. These patients did not enter the study. The remaining patients presenting with acute pain from a permanent tooth were asked to participate in the study (Fig. 1). Written informed consent was obtained from all patients. The current study protocol was approved by the local ethics committee (KEK-ZH-Nr. 2012-0450) and was conducted in accordance with the Declaration of the World Medical Association. Moreover, it was confirmed that this investigation conformed to STROBE guidelines for observational studies. Patients were excluded from the study if they: (i) were not able to clearly communicate in German or English language, (ii) refused to participate, (iii) refused to be, or could not be (pregnancy) diagnosed radiographically, (iv) were on immunosuppressant or long-term anti-inflammatory medication, or took antibiotics during the last 3 weeks, (v) had already initiated treatment of their pain-causing tooth/condition, (vi) could not be diagnosed clearly, (vii) had a condition that was not due to an endodontic infection, or (viii) were diagnosed with an acute apical abscess.

Checklist for key symptoms and pain assessment

Participants who fulfilled the inclusion criteria were interviewed using a checklist with 11 dichotomous questions with a possible discerning value between SIP and SAP (Table 1). This checklist listed symptoms that have been reported in the endodontic literature [20]. It also contained an assessment of pain intensity and duration. The checklist was originally written in German and translated to English for international patients. It was piloted by the authors of this communication and later filled in by either one of two investigators (DKR and GB). Patients were guided through these questions by one of these two investigators. Patients who could not communicate clearly were excluded. In addition, the pain intensity of the presenting condition was assessed using the numeric rating scale (NRS-11; [21]). The examiner asked the patient to quantify his/her maximum pain intensity within the last 24 h on a scale from 0 to 10. The following anchors were used to describe the rating scale:'0' = no pain/pain free and '10' = worst pain imaginable.

Diagnosis

The clinical evaluation included cold testing with carbon dioxide snow, assessment of tenderness to percussion, tooth mobility, and periodontal probing depths. Moreover, the soft tissues were checked for tenderness to palpation, signs of erythema, and presence of a sinus tract or swelling. The findings were compared to a healthy, contralateral tooth that served as a control. Radiographic examination was performed using single-tooth

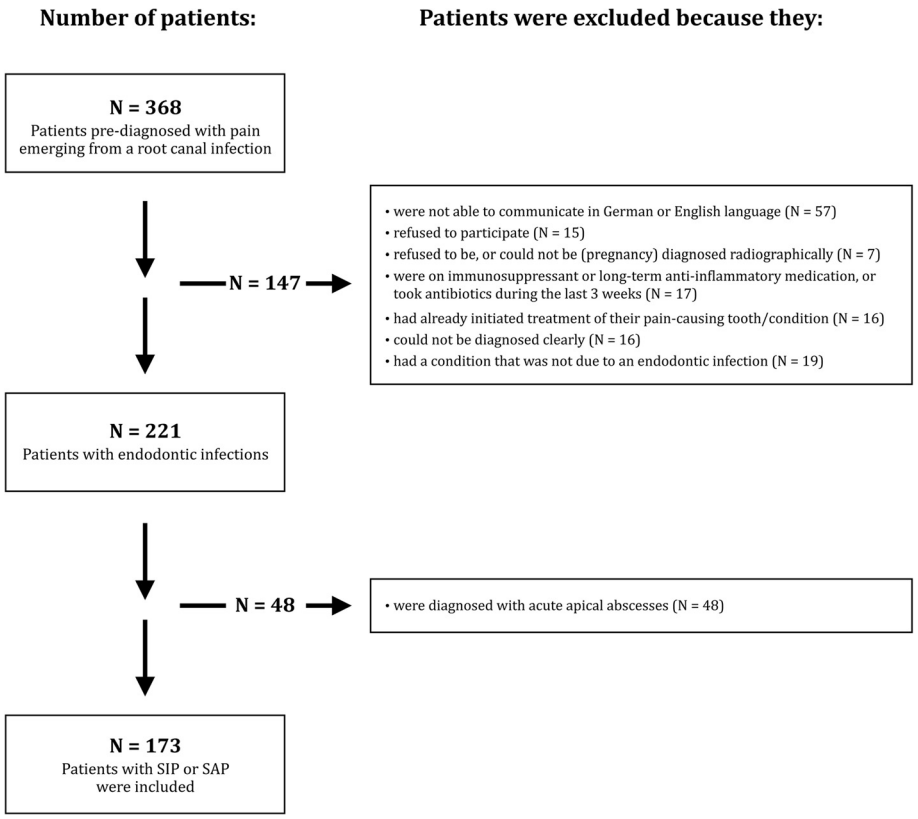

Fig. 1 Flow chart depicting the decisions why patients were excluded from the study. All emergency patients presenting with severe pain originating from a single endodontically involved tooth in the course of 64 weeks were initially considered. Teeth diagnosed with an acute apical abscess were then excluded. *SIP* symptomatic irreversible pulpitis, *SAP* symptomatic apical periodontitis.

radiographs (Digora, Soredex, Tuusula, Finland). The different inflammatory endodontic conditions (SIP, SAP, or AAA) were established according to the recommended diagnostic terminology of the Consensus Conference of the AAE [6]. In a deviation from that nomenclature, however, each tooth was assigned only one main diagnosis (Table 2). The examiner noted this diagnosis, together with relevant data (date, gender, age, analgesics taken in the past 24 h) in an anonymized data sheet.

Statistics and data evaluation

Descriptive statistics included counts and percentages for the questions on pain history. Categorical data was compared between groups using the Chi-squared test. NRS-11 pain levels, which are non-interval ratings, were compared between groups using Mann–Whitney *U* test. The alpha-type error was set at 5 %. To assess the diagnostic value of the 11 symptoms (other than pain) between SAP and SIP, a multiple logistic regression model was fitted to the outcome variable SAP. All of the variables of the checklist were included in order to obtain a predicted probability for SAP for each individual patient. Because some teeth were bridge abutments and the question did thus not make sense, the variable "Tooth feels too high" had 11 missing values. These were multiply imputed with 5 replications. Results of the logistic regression are based on the pooled estimates of the 5 imputed data sets. The area under the receiver operating characteristic (AUC) curve was used to evaluate the discriminative ability of the regression model.

In an alternative approach recursive partitioning was used to construct a decision tree. The focus of the tree was to facilitate the diagnostic decision between SAP and SIP. All variables of the checklist were included in the tree model. All analyses were conducted using R statistical software [22].

Results

Study population and teeth

From January 2013 over a period of 15 month (64 weeks) 368 adult patients attended the dental emergency unit at our institution with severe pain on a permanent tooth. One hundred and forty-seven were not eligible to enter the study because they did not meet the criteria for inclusion (Fig. 1). From the 221 patients with acute pain of endodontic origin, 70 were diagnosed with SIP, 103 with SAP and 48 with AAA. The 48 individuals diagnosed with AAA were also excluded from analysis (Fig. 1). Of

Table 1 History checklist for dental emergency patients asking for symptoms to possibly discern between SIP and SAP

#	Question	Answer options
1	Has the pain interfered with sleep?	y/n
2	Has the pain ever been stronger than during the last 24 h?	y/n
3	Did the pain start less than 1 week ago?	y/n
4	Has the pain been sporadic or constant?	sporadic/ constant
5	Has the pain been localized or radiating?	localized/ radiating
6	How was the main quality of the pain?	dull/sharp
7	Has chewing increased the pain?	y/n
8	Have warm drinks/food increased the pain?	y/n
9	Has cold increased the pain?	y/n
10	Has cold decreased the pain?	y/n
11	Does the affected tooth feel too high?	y/n

SIP symptomatic irreversible pulpitis, *SAP* symptomatic apical periodontitis

the remaining 173 patients diagnosed with either SIP or SAP, the ratio between females to males was 70/103. The average patient age was 40 years and ranged between 18 and 78 years. The ratio between mandibular and maxillary teeth was 107/66. One hundred and thirty of the teeth were molars, 33 premolars and 10 anterior teeth. One hundred and forty-seven of the teeth were multi-rooted, and the remaining 26 were single-rooted. Eleven of the 103 teeth diagnosed with SAP were root-filled. There were no statistical differences regarding tooth types between SIP and SAP in the current cohort ($P > 0.05$).

Pain levels

Pain levels were statistically similar ($P > 0.05$) for both conditions under investigation. Median NRS-11 ratings were 8 for both SAP and SIP, with similar interquartile ranges: 2 for SAP, 1 for SIP. There was also no difference ($P > 0.05$) in pain levels between male and female patients. Eighty-one percent (81 %) of the patients used analgesics within the last 24 h before seeking emergency treatment. There was no statistical difference between the two conditions under investigation in this regard either.

Key symptoms

Assessment of the checklist revealed that large differences in the symptomology of SAP and SIP were found for pain duration, pain on cold, and the feeling that the tooth was too high (Table 3). The prediction model, based on the multiple imputed data set, is summarized in Table 4. Furthermore, the estimated odds ratios and 95 % confidence intervals (CI) are displayed. The discriminative ability of the prediction model resulted in an AUC of 0.796 (95 % CI: 0.728–0.864). The decision tree analysis (Fig. 2) resulted in a tree with splits according to pain on cold, awareness of the tooth feeling too high, and pain duration. The first indicator for SAP was a reported absence of pain to cold stimuli. In teeth that did have a history of pain triggered by cold stimuli, the decision tree correctly identified SAP in 72 % of the teeth that felt too high and had hurt for less than one week. The overall sensitivity of the tree was 95 % and the specificity was 31 %. The positive predictive value was 67 %.

Table 2 Clinical findings used in the current study to differentiate between SIP and SAP

Criterion	SIP	SAP
Sensitivity to carbon dioxide snow	+	-
Radiographically widened ligament space	+/-	+
Periapical radiolucency	-	+/-
Swelling or sinus tract	-	-

SIP symptomatic irreversible pulpitis, *SAP* symptomatic apical periodontitis, + positive response or sign clearly present, +/- mixed response or not present in all cases, - negative response or clearly absent
Note: sensitivity to percussion was not included, as almost all of the acutely painful teeth in this study responded positive to percussion

Discussion

The current study related symptoms to clinical findings. From an immediate treatment planning perspective, SAP is the more critical condition, and its diagnosis should not be missed [16]. It was confirmed that the reported presence or absence of pain to cold stimuli was a first differentiator between the clinical diagnoses of SAP and SIP. Moreover, decision analysis identified additional symptoms associated with a diagnosis of SAP also in teeth with a history of pain to cold stimuli. If these felt too high and had hurt for less than one week, the

Table 3 Descriptive statistics: counts of symptoms in patients diagnosed with SIP ($N = 70$) and SAP ($N = 103$)

Question	SIP	%	SAP	%
Sleep disturbed	56	80.0 %	87	84.5 %
Pain has decreased	11	15.7 %	12	11.7 %
Pain less 1 week	32	45.7 %	72	69.9 %
Constant pain	25	35.7 %	51	49.5 %
Radiating pain	25	35.7 %	26	25.2 %
Sharp pain	38	54.3 %	37	35.9 %
Pain on chewing	49	70.0 %	89	86.4 %
Pain on hot	28	40.0 %	29	28.2 %
Pain on cold	53	75.7 %	37	35.9 %
Cold lessens pain	5	7.1 %	18	17.5 %
Tooth feels high[a]	20	28.6 %	50	48.5 %

SIP symptomatic irreversible pulpitis, *SAP* symptomatic apical periodontitis
[a]This variable had 11 missing values (teeth were bridge abutments)

Table 4 Results of the prediction model for SAP

	Odds ratio	95 % CI
Sleep disturbed	1.0	0.4–2.6
Pain has decreased	0.9	0.3–2.8
Pain less 1 week	2.1	1–4.4
Constant pain	1.6	0.8–3.4
Radiating pain	0.6	0.3–1.5
Sharp pain	0.5	0.2–1
Pain on chewing	2.5	1–6.3
Pain on hot	0.8	0.4–1.6
Pain on cold	0.2	0.1–0.5
Cold lessens pain	0.8	0.2–2.9
Tooth feels high	1.9	0.9–4.3

SAP symptomatic apical periodontitis, *CI* confidence Interval

probability that SAP was diagnosed was still 72 %. A set of questions was thus identified that should be helpful in clinics and for cross-sectional studies to discern between SAP and SIP.

The current study is limited by the fact that data were generated in one single city. The relative frequency of the conditions under investigation is influenced by demographics, the local health care system and socio-economic factors [23]. It would appear that in older studies, SIP was more frequent than SAP [24], while newer investigations including the current work found the opposite [3]. This could be due to the fact that caries, the main cause for symptomatic pulpitis [9, 25], has steadily declined in industrialized countries [26]. Acute forms of apical periodontitis including abscess formation, on the other hand, can occur as late complications in crowned teeth and/or root-filled counterparts [27].

While current pulp tests and two-dimensional radiology are insufficient to determine the exact histological condition of asymptomatic teeth [28–30], the methods that were used in the present study to differentiate between the two acute conditions under investigation can be regarded as sound. The teeth diagnosed with SIP mostly showed a delayed, yet always more pronounced reaction to the cold test compared to healthy counterparts. It has been shown that painful teeth responding with an delayed, but increased and lingering response to the cold test invariably contain a vital pulp or at least vital aspects of the pulp in the apical root canal [25, 28]. In multi-rooted teeth some roots may still contain vital tissue that responds to thermal tests, while in other roots the tissue can be partially necrotic [31]. These vital aspects of the pulp inhibit bacterial infection [32]. Consequently, it is fair to state that acutely painful teeth with positive response to cold test differ from counterparts that test negatively in regard to the level of infection. An intra-operative diagnosis upon entering the pulp space was not performed to differentiate between SIP and SAP.

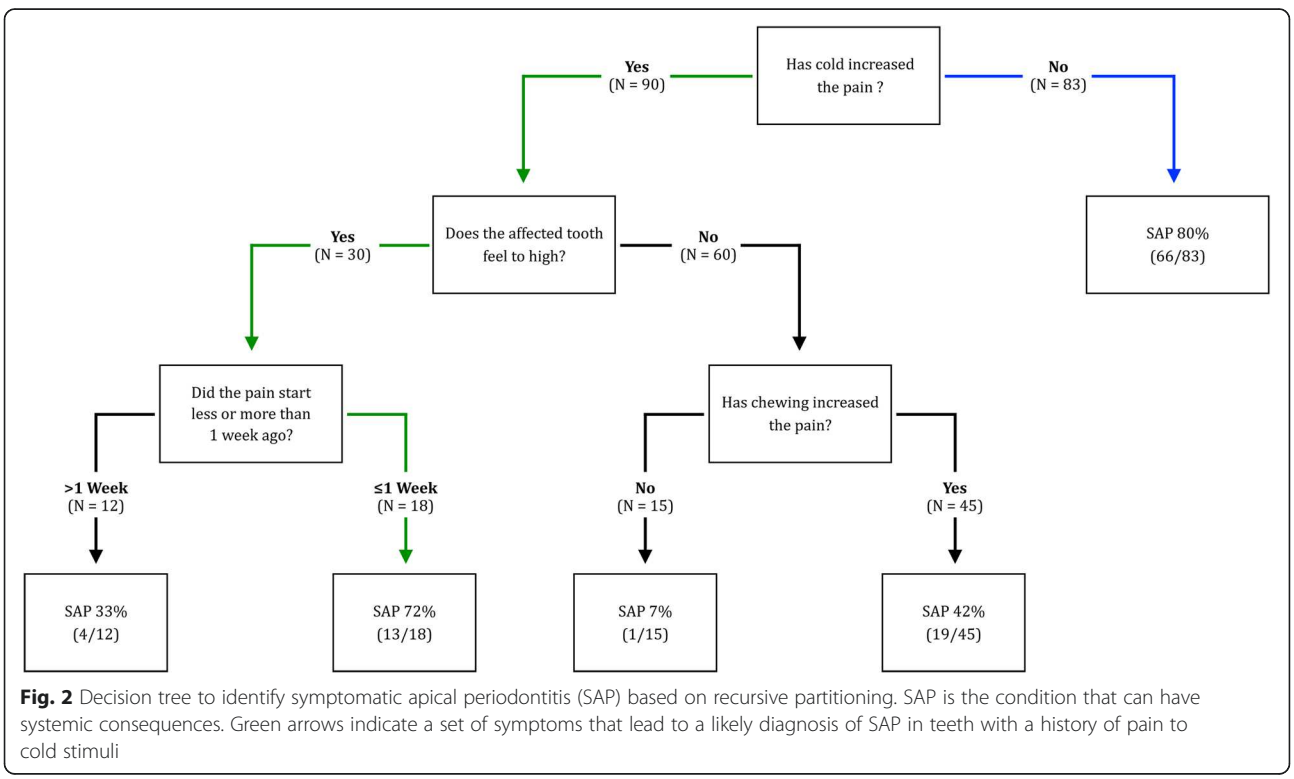

Fig. 2 Decision tree to identify symptomatic apical periodontitis (SAP) based on recursive partitioning. SAP is the condition that can have systemic consequences. Green arrows indicate a set of symptoms that lead to a likely diagnosis of SAP in teeth with a history of pain to cold stimuli

It is known that the intake of analgesics can affect endodontic diagnosis. The pain perception itself can decrease, or the response to clinical test like tooth percussion can be reduced [33]. However, the teeth under investigation were severely painful. Although 81 % ($N = 140$) of the 173 patients included to this study had consumed analgesics within the past 24 h before seeking emergency treatment, 79 % ($N = 136$) of them reported severe pain at levels between 7 and 10 on a NRS-11 scale [21]. Moreover, almost all teeth (92 %; $N = 159$) featured a painful response to percussion. In contrast the influence of analgesics on pulpal sensitivity test has shown to be negligible [34]. Consequently, the influence of analgesics on the diagnostic procedures performed here is expected to be low. It needs to be acknowledged that SIP and SAP are clinically defined separate diagnostic entities based on a set of criteria defined by international consensus (and as such reported in textbooks and major journal articles; [6]). SIP, SAP, and later AAA are biological, dynamically evolving stages of the same underlying bacterial infection. The transition from pulpitis or chronic apical periodontitis to acute apical periodontitis is clinically important, because it marks the point when a spread of the infection from the pulp space to the periapical tissues is about to occur [4]. However, the transitions between these conditions are rarely clear-cut [32]. Furthermore, transitions from one stage to the next can be fast. This is reflected in the current data in that 35.9 % of the patients diagnosed with acute SAP reported a history of sensitivity to cold (Table 3). Almost half of SAP teeth did not show clear apical radiolucencies typical for chronic apical periodontitis (Table 2). This can also be taken as an indicator that the inflammatory conditions in the periapical tissues developed more rapidly than any radiologically discernible bone changes occurred. This is in line with observations published by other authors [10]. Some recent research suggests that bone changes do occur early in the disease process, when the pulp is still vital [35]. These changes, however, are not necessarily detected on single-tooth radiographs [36]. Cone beam computed tomography (CBCT) was shown to be more sensitive in detecting apical disease [36]. The observation on the dynamics of periapical bone changes made here warrants further investigation and verification using CBCT.

Earlier authors concur with the present results in that pain to cold stimuli [28] and the feeling that the affected tooth is too high [13] can help to differentiate between SIP and SAP. However, it has to be cautioned that these authors did not use the current nomenclature of the diseases under investigation, and thus direct comparisons are limited. The current finding that sensitivity to cold is the main indicator for an inflamed vital pulp corresponds to the observations made in the only study on this topic in humans with induced pulpal inflammation [37]. Consequentially, this should further the support of cold testing e.g., carbon dioxide snow being the main clinical test, in conjunction with radiographic images, to diagnose SIP [25]. The patients diagnosed with SAP reported significantly more often the perception that the pain-causing tooth felt too high compared to patients diagnosed with SIP. This observation may be explained by the spread of the inflammation to the periapical ligament. The accumulation of inflammatory exudate may extrude the affected tooth, thus rendering it tender to occlusion [15]. Other common clinical tests were performed in this study, but these were of little value in differentiating between the acute forms of the conditions under investigation. As an example, 159 (92 %) of the 173 teeth in this study were positive to percussion, with no difference between the two conditions. This is in line with published reports: the percussion test has little to no diagnostic value [28, 38]. This is especially the case with painful teeth [39]. Nevertheless, the test is used in the current AAE nomenclature [6]. In accordance with the AAE terminology each tooth that is sensitive to percussion has a periapical diagnosis of "symptomatic apical periodontitis [10, 40]. In the current report, however, the comprehensive diagnosis of the acute forms of SAP before abscess formation versus SIP was based on the thermal responsiveness of the pulp (Table 2).

Numerical rating scales have been validated, and are commonly used for the assessment of pain intensities [7, 41]. The NRS-11 came to use here because of the interview character of this study. In contrast to a visual analogue scale, which is also commonly used in studies on endodontic pain, the NRS can be verbally applied without any visual aids. The 11 potentially differentiating symptoms used in this communication were selected based on the clinical experience of the investigators and common textbook recommendations [20]. They have been assessed, albeit not in the precise formulation attempted here, in various previous studies to differentiate between teeth of different clinical conditions [13, 28]. Pain intensity of endodontically involved teeth causing the patient to seek emergency care was investigated in several previous studies [2, 7, 40, 42]. The current mean NRS-11 pain levels between 7 and 8 correspond well to those measured using a VAS scale on emergency patients in previous studies [7, 42]. The current findings confirm an earlier report in that pain intensity had no differentiating value in the context of acute endodontic conditions [7]. This was not the case for pain *duration* though. Teeth affected by pulpits apparently hurt for more than one week before the pain reached a level that caused the patient to seek emergency care.

The current approach to facilitate the diagnostic decision between SAP and no SAP (SIP) has been two-fold.

Initially, a prediction model including all available clinical information was constructed to assess the discriminative ability of the full checklist as measured with the AUC. The resulting AUC was nearly 80 %, which is an indication that the full model is in some degree valuable for the distinction between patients with SAP and SIP. However, the use of a prediction model based on 11 variables may be difficult to implement in a clinical setting unless a respective computer algorithm will be made available, e.g., in the form of a software application. For that reason, the decision tree analysis, which facilitates the distinction into the groups of SAP and SIP following a specific order of questions/key symptoms, was added in a second step (Fig. 2). The overall sensitivity to detect SAP based on the decision tree was 95 %. However, as is typical for diagnostic decisions with a high sensitivity, the resulting specificity of the tree was lower (31 %). This result demonstrates that the decision tree can be useful for cross-sectional studies. In a clinical setting, the decision tree may be helpful to advise patients during out-of hours calls.

Conclusions

This study confirmed that in severely painful teeth, the most specific single symptom to differentiate between SIP and SAP was pain to cold stimuli. In addition, however, decision analysis identified a set of key symptoms to diagnose SAP also in teeth with a history of pain to cold stimuli. If these felt too high and had hurt for less than one week, the probability that SAP was diagnosed was still 72 %.

Abbreviations

AAA, acute apical abscess; AAE, American Association of Endodontists; CBCT, cone beam computed tomography; NRS, numeric rating scale; SAP, symptomatic apical periodontitis; SIP, symptomatic irreversible pulpitis

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Availability of data and materials

Data supporting the conclusions are included to the article (Tables 3 and 4).

Authors' contributions

DKR conceived this research, was the main investigator and wrote the manuscript. GB helped collecting the data. UH and JMB performed the statistical analysis. TA revised the manuscript and supervised the study. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethical approval and consent to participate

The study protocol was approved by the ethics committee Zurich, Switzerland (KEK-ZH-Nr. 2012-0450). Participation was voluntary and privacy and confidentiality of all study information was maintained. Written informed consent was obtained from all patients.

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Paper 2

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RESEARCH ARTICLE

Biological Markers for Pulpal Inflammation: A Systematic Review

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Abstract

Background and Objective

Pulpitis is mainly caused by an opportunistic infection of the pulp space with commensal oral microorganisms. Depending on the state of inflammation, different treatment regimes are currently advocated. Predictable vital pulp therapy depends on accurate determination of the pulpal status that will allow repair to occur. The role of several players of the host response in pulpitis is well documented: cytokines, proteases, inflammatory mediators, growth factors, antimicrobial peptides and others contribute to pulpal defense mechanisms; these factors may serve as biomarkers that indicate the status of the pulp. Therefore, the aim of this systematic review was to evaluate the presence of biomarkers in pulpitis.

Methods

The electronic databases of MEDLINE, EMBASE, Scopus and other sources were searched for English and non-English articles published through February 2015. Two independent reviewers extracted information regarding study design, tissue or analyte used, outcome measures, results and conclusions for each article. The quality of the included studies was assessed using a modification of the Newcastle-Ottawa-Scale.

Results and Conclusions

From the initial 847 publications evaluated, a total of 57 articles were included in this review. In general, irreversible pulpitis was associated with different expression of various biomarkers compared to normal controls. These biomarkers were significantly expressed not only in pulp tissue, but also in gingival crevicular fluid that can be collected non-invasively, and in dentin fluid that can be analyzed without extirpating the entire pulpal tissue. Such data may then be used to accurately differentiate diseased from healthy pulp tissue. The interplay of pulpal biomarkers and their potential use for a more accurate and biologically based diagnostic tool in endodontics is envisaged.

Introduction

The dental pulp is equipped to express numerous mediators of inflammation, which can combat irritating factors [1–4]. Its mechanistic response begins with vascular changes mediated by Toll-like receptors (TLR) 4/2-positive cells and includes release of measurable inflammatory mediators such as IL-8, IL-6, IL-1 and others [4–7]. Under normal physiologic conditions (left in Fig 1), the vasculature consists of central vessels that branch out into a plexus towards the periphery and specifically the pulp horns. An important difference from soft tissue-enclosed portions of the body is that dental hard tissues enclose the pulp creating a low compliance environment. Dental blood vessels are mainly under control by local metabolites and less by sympathetic innervation. The main cellular components of the pulp are peripherally located odontoblasts and stromal fibroblasts. There are also undifferentiated mesenchymal cells found mainly in the paravascular niche and immune cells (Fig 1). In health, neutrophils predominate but dendritic cells and occasional macrophages are also found.

Inflammation of the dental pulp (pulpitis) has been viewed as a tightly regulated sequence of vascular and cellular events mediated by molecular factors [8]. Pulpitis is typically caused by an opportunistic infection of the pulp space by commensal oral microorganisms [9]. The most common route of entry for the microorganisms is dental caries. Other potential pathways for pulpal microbial infection include trauma, dentinal cracks, exposed dentinal tubules or the main apical foramen [10]. Cells in human dental pulp that express TLR contribute trigger immune responses to microorganisms and their by-products [2–4]. This group includes odontoblasts [11], endothelial cells [12] as well as macrophages and dendritic cells [13]. Some of these cells may form mechanical barriers (i.e. odontoblasts), detect and transmit sensations (nerve fibers) or differentiate (i.e. dental pulp stem cells) to limit infection, signal injury and promote repair, respectively.

Based on the patients' signs, symptoms, and examination, four clinical pulpal conditions are described: normal, reversibly inflamed, irreversibly inflamed or necrotic [14]. Histology represents the gold standard to determine the inflammatory state of pulp tissue [15, 16]; however, it is generally agreed that histologic and clinical classification of pulpal diagnosis still needs to be improved and refined. Normal and necrotic pulps have straightforward histological presentation. The conundrum lies in differentiating reversible and irreversible pulpitis.

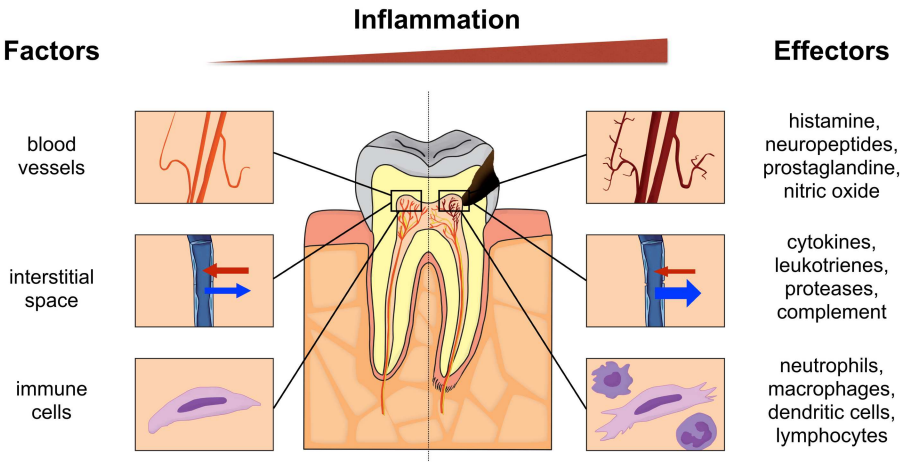

Fig 1. Schematic illustration of a tooth with a healthy pulp (left panel) and an inflamed pulp (right panel) subjacent to a caries lesion. Involved factors and biological effectors are indicated for both pulpal conditions.

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Based on histological reports, reversible pulpitis is characterized by the absence of bacteria and by localized coagulation and liquefaction necrosis immediately surrounding the irritant, whereas irreversible pulpitis is characterized by the presence of the bacteria or their by-products in the dental pulp and by preponderance of acute inflammatory cells predominantly neutrophils in the tissue beneath the lesion suggesting chemotactic activity. Lysosomal enzymes discharged by neutrophils result in widespread tissue damage and suppuration [16–18]. Acute pulpitis (reversible, and irreversible) can be an extremely painful condition and is believed to be one of the main causes for patients to seek emergency dental treatment during or after office hours [19, 20]. The main clinical difference between reversible and irreversible pulpitis is in the pulp's response to thermal stimulus. Reversible pulpitis presents an exaggerated yet non-lingering response to cold stimulus. Irreversible pulpitis on the other hand is characterized by constant, spontaneous pain with exaggerated and lingering response to cold stimulus. However, forty percent of teeth with irreversible pulpitis can be painless [21]. In reversible pulpitis, the pulp is expected to recover after removal of the causative stimulus. In contrast, if the pulp is irreversibly inflamed, healing is not expected and pulpectomy (i.e., full removal of the dental pulp) is indicated.

The succession of signaling events resulting from dental pulp stimulation by microorganisms to the release of an array of immune mediators that in turn may cause pulpal or odontogenic pain, pulpitis, or in advanced stages, pulpal necrosis and finally apical periodontitis have been well described in the past [4–7]. Detailed discussion of these mechanisms is beyond the scope of this article.

Currently, diagnostic procedures that aim to assess pulpal inflammation involve case history, as well as clinical and radiographic examination. Clinical examination includes different procedures such as inspection, pulp sensitivity to thermal or electric stimuli, and pain on palpation or percussion. These procedures apparently did not change much in the last century [22]. However, the validity of the currently employed clinical tests to determine the actual or histopathological status of the pulp remains controversial [15]. A recently performed literature review summarized the available information on the diagnostic accuracy of signs/symptoms and current tests used to determine the condition of the pulp [23]. These authors concluded that the overall evidence was insufficient to support the accuracy of such test, even if the tests are combined. Hence, the current diagnostic procedures do not reliably identify the inflammatory status of the pulp. This is particularly unfortunate since decision making in this field, for example differentiation between vital pulp therapy and root canal treatment, critically depends on an accurate pulpal diagnosis.

According to the National Library of Medicines, the medical subject heading term (MeSH term) definition for a biological marker is a measurable and quantifiable biological parameter that serves as an indicator for health- and physiology-related assessments. Molecules expressed in the cascade of tissue inflammation may serve as (diagnostic) biomarkers for the presence of inflammation. Some research suggests that the dental pulp is not an isolated entity in an encased, solid environment but a reactive tissue that extends its biological products into the outside environment [24, 25]. In fact, studies have shown that pulpal events can be reflected through measurable levels of protein markers that correlated with pulpal symptoms in pulpal blood [26], dentinal fluid [27], periapical fluid [28], and gingival crevicular fluid (GCF; [1, 29]).

In the field of periodontology, biomarkers in oral fluids/saliva or gingival crevicular fluid are used to detect the occurrence and progression of periodontitis [30, 31]. For example, matrix metalloproteinases (MMPs) such as MMP-8 and -9 have been shown to be central biomarkers of soft tissue breakdown in periodontal pockets [32]. Periodontal and pulpal inflammation shares certain features: initially, both exhibit soft-tissue inflammation caused by microbial infection. At a later stage, these pathologic processes culminate in bone resorption

(vertical bone-loss or apical periodontitis, respectively). It is therefore possible that both pathoses may express the same biomarkers. In this regard, MMPs were shown to be potential biomarker for both pulpal [33] and periodontal disease [32]. However, the application of molecular diagnostics in pulpal disease is as yet not used for clinical decision-making [34].

Previous studies have investigated the molecular regulatory pathways of pulpal inflammation employing explanted cell cultures *in vitro* [35–37]. However, the extrapolation of such results to the clinical situation is difficult, perhaps due to the reductionist nature of such experiments. *In vivo*, the presence of other cellular players (e.g. immune cells), inhibitory proteins (e.g. protease inhibitors) and other molecules that modify the inflammatory response may present a completely different inflammatory response and consequently, a different clinical outcome compared with what may be suggested by *in vitro* experimental results. Studies reporting clinical samples for the presence of potential biomarkers for pulpal inflammation are still on the rise. The clinical importance of identifying these biomarkers that can be used to diagnose or to stage pulpal inflammation warrants not only additional studies but also a critical or systematic review and analysis of published reports. Therefore, the aim of this paper is to systematically review the currently available information on biomarkers that were identified from pulp tissues diagnosed as normal or inflamed.

Systematic Review

Eligibility Criteria and Literature Search

This systematic review was prepared in accordance with PRISMA (S1 Table) [38]. Studies were eligible for inclusion to the review that clinically and/ or histologically differentiate between a healthy and a irreversibly inflamed pulp in permanent human teeth, and analyzed interstitial/ dentinal liquor, gingival crevicular fluid, pulpal tissue, dentin fluid or apical blood for the presence of a biological marker. A biological marker is defined as measurable and quantifiable biological molecule that theoretically can be present in those substrates and might serve as an indicator for a healthy or diseased pulp (adapted from MeSH Unique ID: D015415). An electronic search strategy with combined keywords and indexing vocabulary (MeSH terms) was conducted in the Medline database of the US National Library of Medicine employing the OvidSP interface. We used the following search terms and other subject headings: 'pulpitis', 'acute pulpitis', 'irreversible pulpitis', 'painful pulpitis', 'biological markers', 'inflammation mediators', 'dentinal fluid', and 'gingival crevicular fluid'. S2 Table lists the detailed search strategy performed in Medline. The same electronic search strategy was used in Biosis (OvidSP), the Cochrane library (Wiley), Embase (http://www.embase.com) and the Web of Science (Thomson Reuters). The last date entered was February 19, 2015. No language restrictions were imposed and all articles were included from the inception of the respective database (S3 Table). To ensure the completeness of the search, one reviewer (DRK) conducted a thorough search of the bibliographies of all included studies.

Study Selection and Quality Assessment

The search and selection process is summarized in Fig 2 [38]. A pool of 1733 records was initially identified using the electronic search strategy and other sources. After removal of duplicates, 851 records remained. Two reviewers (DKR and JCG) independently screened the titles and abstracts of the references collected. Communications not related to the topic were discarded (n = 695). Communications deemed appropriate by one of the reviewers were assigned for full text evaluation. One hundred and fifty-six records were identified using this approach and reviewed as full texts. Articles were collected and evaluated independently by both reviewers. Non-English abstracts or manuscripts were translated with the help of translators. Further

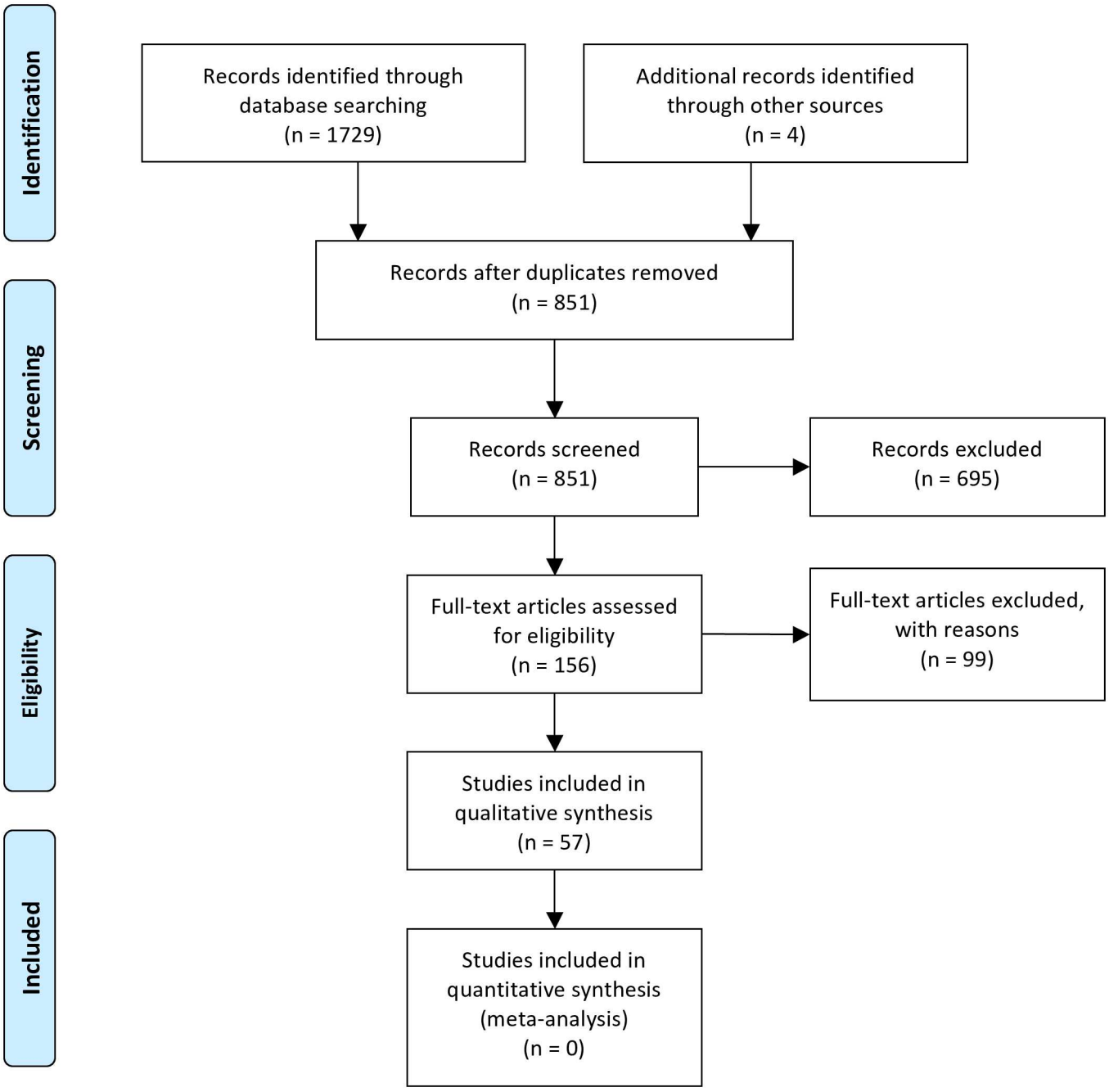

Fig 2. PRISMA flowchart depicting the systematic selection and exclusion of articles related to the topic. A detailed description of the excluded articles with the respective reasons for exclusion is presented in the running text and S4 Table. *From*: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). *P*referred *R*eporting *I*tems for Systematic Reviews and *M*eta-*A*nalyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi: 10.1371/journal.pmed1000097 **For more information, visit** www.prisma-statement.org.

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articles (n = 99) were excluded for one of the following reasons: i) studies not on human teeth, ii) cell culture study only, iii) no potential biomarker was investigated or the study was off topic, iv) no clear distinction between reversible, irreversible or necrotic pulp, v) studies rather on histologic features or presence of cells, bacteria or viruses than on quantification of a biomarker, vi) review articles, editorials, comments, abstract only or case reports (S4 Table). In

case of disagreement consensus was achieved through discussion by third party arbitration (OAP). Articles where no exclusion criteria applied were included to the review. There was 94.2% agreement prior to arbitration between both reviewers and finally 57 publications were included to the review. The included articles were written in English (n = 54) or Chinese (n = 3) language.

Quality Assessment

The quality of the included studies was assessed using a modification of the Newcastle-Ottawa-Scale (NOS; [39, 40]). The NOS rates the 3 study domains 'selection', 'comparability' and 'outcome'. Each positive rating was awarded with a star. The parameters recorded for 'selection' were: selection of the cohort (gender and age distribution reported) and condition of the cohort (general health and medication reported). The parameters recorded for 'comparability' were: diagnostics of cases and controls (anamnesis, clinical and radiological inspection described in sufficient detail), histological confirmation of the diagnosis performed (yes/no), quality of the controls (control sample from the same patient as the case sample) and the ratio of the group size (cases:controls $\geq$ 1:2). The parameters recorded for 'outcome' were: reported blinding to the case/control status (yes/no) and that the same tests were performed with the cases and control samples (yes/no). Consequently two stars could be awarded for 'selection', four stars for 'comparability' and two stars for 'outcome'. A value of '0' represents the lowest study quality and '8' the highest possible quality rating of the modified NOS.

Data Extraction and Statistical Analysis

Quantitative data were collected from all studies included to the review. An electronic protocol for data extraction was defined and piloted on several manuscripts before final completion. Relevant information regarding reference name, publication date, substrate analyzed for the presence of a biological marker, how was the substrate collected, number of specimens in experimental group and control group, was the substrate pooled before analysis (yes/no), name of the biological marker under investigation, what type of molecule is the biological marker, what general function serves the biological marker, what was the molecular expression level of the biological marker, which analyte was evaluated for the presence of the biological marker, which method was used for analysis, and were statistically significant differences between specimens of the irreversible pulpitis group compared to the control group (healthy pulp) reported (yes/no), were collected. The synthesis of the data is presented in a descriptive manner. Moreover, descriptive statistics were applied when deemed appropriate (JMP 10.0.0, SAS Institute, Cary, N.C., USA).

Results

Study Characteristics and Quality Assessment

The studies excluded during full text evaluation (N = 99; Fig 2) are presented in S4 Table. The studies included in the review are listed in Tables 1 and 2. Due to the heterogeneous nature of the studies it was not possible to perform meta-analysis on their outcome. The quality ratings of the included studies according to the modified NOS are presented, along with the full reference, in S5 Table. The average quality score was 3.9 ± 1.1 (mean ± SD). Fig 3 provides an overview on the total ratings for the respective parameters for the study domains selection, comparability and outcome. Weaknesses were noticed for the parameters selection of the cohort, quality of the controls and reported blinding to the case/control status.

Table 1. Studies assessing pulp tissue for the presence of biomarkers associated with pulpal condition.

Reference	*n* per group (irreversibly inflamed/ non-inflamed)	Biomarker	Function	Target	Analyte	Method	Significant difference between groups
Cytokines							
Zehnder et al. 2003 [48]	11/13	IL-1α	Regulates immune and inflammatory reactions; stimulates bone resorption	mRNA	PTS	RT-PCR	n
Abd-Elmeguid et al. 2013* [49]	12/30			Protein	PTS	Multiplex assay	y
Abd-Elmeguid et al. 2013* [49]	12/30	IL-1rα	Acute phase protein that increases neutrophil presence of	Protein	PTS	Multiplex assay	y
Zehnder et al. 2003 [48]	11/13	IL-1β	Regulates immune and inflammatory reactions; stimulates bone resorption	mRNA	PTS	RT-PCR	n
Paris et al. 2009 [50]	10/7			mRNA	PTS	RT-PCR	y
Silva et al. 2009* [51]	5/5			Protein	PTS	ELISA	n
Abd-Elmeguid et al. 2013* [49]	12/30			Protein	PTS	Multiplex assay	y
Rauschenberger et al. 1997* [52]	15/17	IL-2	Regulates activities of leukocytes	Protein	PTS	ELISA	y
Anderson et al. 2002 [53]	32/24			Protein	PTS	ELISA	n
Abd-Elmeguid et al. 2013* [49]	12/30	IL-4	Key regulator in humoral and adaptive immunity; stimulates activated B cells, T-cell proliferation, and the differentiation of B-cells into plasma cells	Protein	PTS	Multiplex assay	y
Zehnder et al. 2003 [48]	11/13	IL-6	Regulator of T- and B-cell growth, acute phase protein production	mRNA	PTS	RT-PCR	y
Abd-Elmeguid et al. 2013* [49]	12/30			Protein	PTS	Multiplex assay	y
Abd-Elmeguid et al. 2013* [49]	12/30	IL-7	Stimulates proliferation and maturation of B and T cells	Protein	PTS	Multiplex assay	y
Huang et al. 1999* [54]	14/15	IL-8	Recruitment and activation of neutrophils	Protein	PTS	ELISA	y
Zehnder et al. 2003 [48]	11/13			mRNA	PTS	RT-PCR	y
Silva et al. 2009* [51] *	5/5			Protein	PTS	ELISA	n
Abd-Elmeguid et al. 2013* [49]	12/30			Protein	PTS	Multiplex assay	y
Abd-Elmeguid et al. 2013* [49]	12/30	IL-12p40	Subunit of IL-12; acts on T- and natural killer cells	Protein	PTS	Multiplex assay	y
Abd-Elmeguid et al. 2013* [49]	12/30	IL-13	Mediator of allergic inflammation and disease	Protein	PTS	Multiplex assay	y
Abd-Elmeguid et al. 2013* [49]	12/30	IL-15	Induces proliferation of natural killer cells	Protein	PTS	Multiplex assay	n
Zehnder et al. 2003 [48]	11/13	IL-18	Pro-inflammatory cytokine involved in cell mediated immunity	mRNA	PTS	RT-PCR	y
Pezelj-Ribaric et al. 2002 [55]	19/18	TNF-α	Delays neutrophil apoptosis	Protein	PTS	ELISA	y
Kokkas et al. 2007 [43]	6/6			mRNA	PTS	RT-PCR	y
Keller et al. 2009 [56]	5/5			mRNA	PTS	RT-PCR	y
Paris et al. 2009 [50]	10/7			mRNA	PTS	RT-PCR	y
Abd-Elmeguid et al. 2013* [49]	12/30			Protein	PTS	Multiplex assay	y

(*Continued*)

Table 1. (*Continued*)

Reference	*n* per group (irreversibly inflamed/ non-inflamed)	Biomarker	Function	Target	Analyte	Method	Significant difference between groups
Abd-Elmeguid et al. 2013* [49]	12/30	TNF-β	Mediates a large variety of inflammatory, immunostimulatory, and antiviral responses	Protein	PTS	Multiplex assay	n
Li et al. 2011* [57]	4/4	MIP-1α	Mediate immune responses towards infection and inflammation; activation of granulocytes	mRNA	PTS	RT-PCR	y
Abd-Elmeguid et al. 2013* [49]	12/30			Protein	PTS	Multiplex assay	y
Abd-Elmeguid et al. 2013* [49]	12/30	MIP-1β	Mediate immune responses towards infection and inflammation; activation of granulocytes	Protein	PTS	Multiplex assay	y
Nakanishi et al. 2005* [58]	8/5	MIP-3α	Chemoattractant for lymphocytes and neutrophils	Protein	FPT	IHC	n/a
Nakanishi et al. 2005* [58]	8/5	CCR6	MIP-3α Receptor on memory T-cells, dendritic cells and Th17 cells	Protein	FPT	IHC	n/a
Abd-Elmeguid et al. 2013* [49]	12/30	TGF-α	Induces epithelial development and wound healing	Protein	PTS	Multiplex assay	y
Piattelli et al. 2004* [59]	20/23	TGF-β1	Modulates pro-inflammatory cytokine production, inhibits mitogenic effects of IL-2 on T and B lymphocytes, blocks activity of other immunocompetent cells	Protein	FPT	IHC	y
Adachi et al. 2007* [60]	9/4	CXCL10	Chemoattractant for monocytes/ macrophages, T cells, NK cells, and dendritic cells	Protein	PTS	RT-PCR	y
	5/4			Protein	FPT	IHC	n/a
Jiang et al. 2008* [61]	6/5	SDF-1	Chemotactic for lymphocytes	mRNA	PTS	RT-PCR	y
	4/4			Protein	FPT	IHC	n/a
Huang et al. 2009* [62]	15/15	Oncostatin M	Involved in hematopoiesis, tissue remodelling processes and inflammation	mRNA	PTS	RT-PCR	y
				Protein	FPT	IHC	y
Abd-Elmeguid et al. 2013* [49]	12/30	GM-CSF	Stimulates production of granulocytes and monocytes	Protein	PTS	Multiplex assay	y
Abd-Elmeguid et al. 2013* [49]		GRO	Neutrophil chemoattractant. Involved in angiogenesis, inflammation, wound healing and tumorigenesis	Protein	PTS	Multiplex assay	y
Abd-Elmeguid et al. 2013* [49]		MCP-1	Chemoattractant for monocytes, recruits memory T cells, and dendritic cells to the sites of inflammation	Protein	PTS	Multiplex assay	y
Abd-Elmeguid et al. 2013* [49]		MCP-3	Chemoattractant for monocytes; regulates macrophage function	Protein	PTS	Multiplex assay	y
Abd-Elmeguid et al. 2013* [49]		MDC	Chemotactic for monocytes, dendritic cells and natural killer cells	Protein	PTS	Multiplex assay	y
Abd-Elmeguid et al. 2013* [49]		INF-α	Antiviral agents, modulate functions of the immune system	Protein	PTS	Multiplex assay	y
Abd-Elmeguid et al. 2013* [49]		G-CSF	Stimulates proliferation and differentiation of granulocytes	Protein	PTS	Multiplex assay	y
Abd-Elmeguid et al. 2013* [49]		Eotaxin-1	Recruits eosinophils by inducing their chemotaxis	Protein	PTS	Multiplex assay	y

(*Continued*)

Table 1. (*Continued*)

Reference	*n* per group (irreversibly inflamed/ non-inflamed)	Biomarker	Function	Target	Analyte	Method	Significant difference between groups
Abd-Elmeguid et al. 2013* [49]		flt3ligand	Stimulates proliferation and differentiation of various blood cell progenitors	Protein	PTS	Multiplex assay	y
Abd-Elmeguid et al. 2013* [49]		Fractalkine	Chemoattractant for T cells and monocytes; promotes strong adhesion of leukocytes to activated endothelial cells	Protein	PTS	Multiplex assay	y
Abd-Elmeguid et al. 2013* [49]		CD40L	Co-stimulatory molecule for T cells; promotes B cell maturation and function	Protein	PTS	Multiplex assay	n
Abd-Elmeguid et al. 2013* [49]		sIL-2rα	Receptor that mediates IL-2 activities; increased levels biological fluids correlate with increased immune system activation	Protein	PTS	Multiplex assay	n
Abd-Elmeguid et al. 2013* [49]		IP-10	Chemoattractant for monocytes/ macrophages, T cells, NK cells, and dendritic cells	Protein	PTS	Multiplex assay	n
Abd-Elmeguid et al. 2013* [49]		PDGF-AA	Receptor that regulates cell proliferation, cellular differentiation, cell growth and development	Protein	PTS	Multiplex assay	n
Abd-Elmeguid et al. 2013* [49]		PDGF-AB/BB	Receptor that regulates cell proliferation, cellular differentiation, cell growth and development	Protein	PTS	Multiplex assay	n
Abd-Elmeguid et al. 2013* [49]		RANTES	Chemoattractant for leukocytes to inflammatory sites; proliferation and activation of natural-killer cells	Protein	PTS	Multiplex assay	n
Abd-Elmeguid et al. 2013* [49]		Osteocalcin	Regulation of bone mineralization	Protein	PTS	Multiplex assay	y
				Protein	FPT	IHC	n/a
Proteases and other enzymes							
Gusman et al. 2002 [63]	17/18	MMP-1	Regulator of connective tissue remodeling	Protein	PTS	ELISA	*Not detected*
Gusman et al. 2002 [63]	17/18	MMP-2	Hydrolysis of intercellular matrix	Protein	PTS	ELISA	y
Accorsi-Mendonca et al. 2013 [64]	10/10			Protein	PTS	Zymography	y
Accorsi-Mendonca et al. 2013 [64]	10/10	pro-MMP-2	Pro-form of MMP-2	Protein	PTS	Zymography	n
Gusman et al. 2002 [63]	17/18	MMP-3	Hydrolysis of intercellular matrix	Protein	PTS	ELISA	y
Tsai et al. 2005* [65]	14/14			mRNA	PTS	RT-PCR	y
				Protein	FPT	IHC	y
Gusman et al. 2002 [63]	17/18	MMP-9	Hydrolysis of intercellular matrix; regulatory factor for neutrophil migration across basement membrane	Protein	PTS	ELISA	y
Suwanchai et al. 2012 [66]	7/18			Protein	PTS	Western Blot	y
Accorsi-Mendonca et al. 2013 [64]	10/10			Protein	PTS	Zymography	n/a
Huang et al. 2005* [67]	17/13	t-PA	Involved in soft-tissue breakdown; catalyzes the conversion of plasminogen to plasmin	mRNA	PTS	RT-PCR	y

(*Continued*)

Table 1. (*Continued*)

Reference	*n* per group (irreversibly inflamed/ non-inflamed)	Biomarker	Function	Target	Analyte	Method	Significant difference between groups
				Protein	FPT	IHC	n/a
Huang et al. 2007* [68]	22/9			Protein	PTS	Zymography	y
	22/9			Protein	PTS	ELISA	y
Ge et al. 1996 [69]	12/9	SOD	Antioxidant	Protein activity	PTS	Enzyme assay	y
Tulunoglu et al. 1998 [70]	10/7			Protein activity	PTS	Enzyme assay	n
Bodor et al. 2007 [71]	16/10	Cu, ZN-SOD	Protection against reactive oxygen species	mRNA	PTS	RT-PCR	y
Varvara et al. 2005 [72]	13/12			Protein activity	PTS	Enzyme assay	y
Bodor et al. 2007 [71]	16/10	Mn-SOD	Protection against reactive oxygen species	mRNA	PTS	RT-PCR	y
Ge et al. 1996 [69]	12/9	MDA	Oxidative stressor	Protein activity	PTS	Enzyme assay	y
Cootauco et al. 1993* [73]	5/8	Elastase	Cleavage of elastin, collagen, proteoglycans	Protein	FPT	IHC	y
		Cathepsin-G	Proteolysis	Protein	FPT	IHC	y
Spoto, Fioroni, Rubini, Tripodi, Di Stilio, et al. 2001 [74]	10/10	Alkaline phosphatase	Hydrolysis of phosphate ester-bonds	Protein activity	PTS	Enzyme assay	n
Spoto, Fioroni, Rubini, Tripodi, Perinetti, et al. 2001 [75]	20/20	Aspartate Aminotransferase	Catalyzes transfer of aminotransferase amino group of aspartate to α-ketoglutarat	Protein activity	PTS	Enzyme assay	n
Esposito, Varvara, Caputi, et al. 2003 [76]	15/18	Catalase	Catalyzes the breakdown of hydrogen peroxide	Protein activity	PTS	Enzyme assay	y
Esposito, Varvara, Murmura, et al. 2003 [77]	12/11			Protein activity	PTS	Enzyme assay	y
da Silva et al. 2008* [78]	6/6	NADPH-diaphorase	Detoxification to produce ROS	Protein	FPT	IHC	y
Di Nardo Di Maio et al. 2004* [79]	10/10	eNOS	Nitric oxide synthase	mRNA	PTS	RT-PCR	y
				Protein	PTS	Western blot	y
				Protein	FPT	IHC	y
	10/10	iNOS	Nitric oxide synthase	mRNA	PTS	RT-PCR	y
				Protein	PTS	Western blot	y
				Protein	FPT	IHC	y
Spoto, Ferrante, et al. 2004 [80]	6/12	cGMP PDE	Hydrolysis of cyclic nucleotide	Protein activity	PTS	Enzyme assay	y
Spoto, Menna, et al. 2004 [81]	6/12	cAMP PDE	Hydrolysis of cyclic nucleotide	Protein activity	PTS	Enzyme assay	y
Accorsi-Mendonca et al. 2013 [64]	10/10	TIMP-2	Inhibits MMP-2	Protein	PTS	ELISA	y
		MPO	Generation of reactive oxygen species	Protein activity	PTS	Enzyme assay	y
Inflammatory mediators							
Bolanos and Seltzer 1981 [82]	17/7	cAMP	Activation of protein kinases	Protein	PTS	RIA	n

(*Continued*)

Table 1. (*Continued*)

Reference	*n* per group (irreversibly inflamed/ non-inflamed)	Biomarker	Function	Target	Analyte	Method	Significant difference between groups
		cGMP	Activation of protein kinases	Protein	PTS	RIA	n
Cohen et al. 1985 [47]	13/20	PGE2	Multiple pro-inflammatory and immunomodulatory effects	Protein	PTS†	RIA	y
		PGF2α	Multiple pro-inflammatory and immunomodulatory effects	Protein	PTS†	RIA	y
Cootauco et al. 1993* [73]	5/8	α-2M	Neutralization of proteinases	Protein		IHC	n/a
Dong et al. 1999 [83]	9/11	6-K-PGF1α	Vasodilators; inhibits the aggregation of blood platelets; involved in inflammation	Protein	PTS	RIA	y
		TXB2	Involved in platelet aggregation, vasoconstriction and reproductive functions	Protein	PTS	RIA	y
Khabbaz et al. 2001 [84]	15/5	Endotoxins	Induces strong immune response	Protein activity	PTS	LAL	y
Nakanishi et al. 2001* [85]	10/5	COX-2	Prostaglandin synthesis	Protein		IHC	n/a
Guven et al. 2007* [86]	12/12			Protein	FPT	IHC	n/a
Awawdeh et al. 2002 [87]	46/20	Substance P	Vasoactive mediator, immune mediator	Protein	PTS	RIA	y
Caviedes-Bucheli et al. 2006 [88]	6/6			Protein	PTS	RIA	y
Awawdeh et al. 2002 [87]	46/20	Neurokinin A	Generates three different preprotachykinins	Protein	PTS	RIA	y
Caviedes-Bucheli et al. 2006 [88]	6/6			Protein	PTS	RIA	y
Awawdeh et al. 2002 [87]	46/20	CGRP	Vasodilation and increased microvascular permeability	Protein	PTS	RIA	y
Caviedes-Bucheli et al. 2004 [89]	5/5			Protein	Pulp cells in suspension	Flow cytometry	y
Caviedes-Bucheli et al. 2005 [90]	6/4			Protein	PTS	RIA	y
Caviedes-Bucheli et al. 2006 [88]	6/6			Protein	PTS	RIA	y
Caviedes-Bucheli et al. 2006 [88]	6/6	Neuro-peptide Y	Potent vasoconstrictor, parasympathetic nervous system	Protein	PTS	RIA	y
		VIP	Vasodilator, parasympathetic nervous system	Protein	PTS	RIA	n
da Silva et al. 2008* [78]	6/6	NOD2	Involved in host response against bacteria	mRNA	PTS	RT-PCR	y
Keller et al. 2009 [56]	5/5			mRNA	PTS	RT-PCR	y
Growth Factors							
Artese et al. 2002* [91]	25/25	VEGF	Stimulates vasculogenesis and angiogenesis	Protein	FPT	IHC	n/a
Guven et al. 2007* [86]*	12/12			Protein	FPT	IHC	n/a
Abd-Elmeguid et al. 2013* [49]	12/30			Protein	PTS	Multiplex assay	n

(*Continued*)

Table 1. (*Continued*)

Reference	*n* per group (irreversibly inflamed/ non-inflamed)	Biomarker	Function	Target	Analyte	Method	Significant difference between groups
Abd-Elmeguid et al. 2013* [49]	12/30	FGF	Involved in angiogenesis, wound healing, embryonic development and various endocrine signaling pathways	Protein	PTS	Multiplex assay	n
Antimicrobial peptides							
Paris et al. 2009 [50]	10/7	hBD-1	Activates the innate and adaptive immune system. Chemotactic for monocytes, T-lymphocytes, dendritic cells and mast cells	mRNA	PTS	RT-PCR	y
		hBD-2		mRNA	PTS	RT-PCR	n
		hBD-3		mRNA	PTS	RT-PCR	n
		hBD-4		mRNA	PTS	RT-PCR	y
Others							
Caviedes-Bucheli et al. 2007 [92]	5/5	Substance P receptor	Vasoactive mediator, immune mediator	Protein	PTS	RIA	y
Caviedes-Bucheli, Moreno, et al. 2008 [93]	13/13	AAMø CD163 + expressing CGRPr	Alternatively activated polarized monocyte/macrophage; different phenotype compared to the classical ones. Then expressing CD163+	Protein	Pulp cells in suspension	Flow cytometry	*See text*
Suwanchai et al. 2012 [66]	7/18	NaV 1.8	Initiation and propagation of action potentials; involved in pain perception	Protein	PTS	Western blot	y
		NaV 1.9	Initiation and propagation of action potentials; involved in pain perception	Protein	PTS	Western blot	y
Zhong et al. 2012 [94]	18/12	miRNAs	Regulators of post-transcriptional gene expression in biological processes like inflammation, immune response, and osteoclastic bone resorption	mRNA	PTS	Microarray	*See text*
Dong et al. 2013* [95] *	21/12	EphA7	Involved in embryonic development, angiogenesis, tumorigenesis, inflammation & pain	Protein	FPT	IHC	y
				mRNA	PTS	RT-PCR	y

* Pulpal inflammation confirmed histologically;

† Substrate pooled before analysis;

y: Yes; n: No; n/a: Not applicable.

Analytes were mostly either pulp tissue supernatant (PTS) or fixed pulp tissue (FPT). One study used pulp cells in suspension, another one pulpal fluid. Analytical methods used included reverse transcription polymerase chain reaction (RT-PCR), multiplex assay, microarray, Western Blot, radioimmunoassay (RIA), immunohistochemistry (IHC), enzyme-linked immunosorbent assay (ELISA), zymography, flow cytometry, limulus amoebocyte assay (LAL), and specific enzyme assays.

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Tissues Studied

Eighty-eight percent of the studies included (50/57; Table 1) analyzed pulp tissue for the presence of a biomarker either collected via pulpectomy (N = 5), tooth extraction and fracturing (N = 25), or a combination of both (N = 20). Twelve percent (7/57; Table 2) of the studies included analyzed substrates other than pulp tissue: pulpal blood (N = 2), peripheral blood serum (N = 1), GCF (N = 1), dentinal fluid (N = 1), or extracellular pulpal fluid (N = 2). Pulpal blood, GCF and dentinal fluid were collected using absorbable membranes, blood serum via peripheral blood collection and extracellular pulpal fluid by inserting microdialysis membranes into vital pulp tissue [41, 42]. Eighty-two percent of the studies analyzing pulp tissue

(41/50) used tissue collected from extracted healthy, non-carious permanent, or wisdom teeth as their control. Fourteen percent (7/50) used healthy pulp tissue collected via pulpectomy because of elected root canal treatment for prosthetic reasons as their control. One study used tissues from extraction and pulpectomy as control [43], another one did not state precisely how they collected control tissue [2]. Of the 7 studies evaluating substrates other than pulp tissue, two sampled blood [44, 45], and another one extracellular fluid [41] from healthy teeth that were assessed but subsequently planed for extraction because of prosthetic or orthodontic reasons as control. One study sampled venous (peripheral) blood during pulp inflammation and used a consecutive peripheral blood sample after treatment as control [46]. One further study sampled GCF from healthy contralateral or adjacent teeth as control [1], and another one collected dentinal fluid from non-symptomatic teeth scheduled for replacement of a filling as control [33]. The substrate in one study was pooled before performing the confirmatory test [47].

Confirmatory Tests

Analytical methods used for the assessment of pulp tissue included reverse transcription polymerase chain reaction, multiplex assay, microarray, western blot, radioimmunoassay, immunohistochemistry, enzyme-linked immunosorbent assay, zymography, flow cytometry, limulus amoebocyte assay and specific enzyme assays (Table 1). Pulpal inflammation was confirmed by histology in 42% (21/50; Table 1 and S5 Table) of these studies. Substrates other than pulp tissue were analyzed using radioimmunoassay, enzyme-linked immunosorbent assay, specific serum, or enzyme assays (Table 2). Histology was not used to confirm pulpal diagnosis in those studies. Seventy-four percent of the studies evaluating pulp tissue (37/50) analyzed actual protein expression or protein activity, whereas 16% (8/50) analyzed the pulp tissue on the DNA level. Five studies (10%) analyzed the pulp tissue substrates at both levels (Table 1). All studies evaluating other substrates than pulp tissue evaluated protein expression or protein activity (Table 2).

Markers Studied

Pulp tissue was assessed for a total of 89 biological markers. Statistical significant differences between an irreversible inflamed and a healthy pulp could be detected for 64 biological markers (71.9%) by at least one study. Nineteen biological markers showed no statistically significant differences between inflammation and health, whereas 6 biological markers were not evaluated employing statistical tests (Table 1). Substrate other than pulp tissue was evaluated for 16 biological markers. For twelve biological marker (75%) statistical significant differences between irreversible inflammation and health could be detect by at least one study.

Discussion

The results point to a response in pulpitis by immunocompetent tissues that ultimately results in the release of mediators, which in turn trigger a series of inflammatory events and an attempt to initiate repair. Collectively, the data presented here demonstrate the involvement of various TLR-induced chemotactic molecules (i.e. IL-8, CXCL-10, MIP family, GRO, MCP family, RANTES, Eotaxin, IP10, and others).

TLRs have been shown to confer immunocompetence to the dental pulp [56]. They are expressed by both immune and non-immune cells in the pulp including neurons, fibroblasts, endothelial cells, epithelial cells and others, which recognize viral and microbial structures as well as self molecules (such as single stranded RNAs) that may accumulate in non-physiologic amounts or sites during inflammation [96–100].

Table 2. Studies assessing other substrates than pulp tissue for the presence of a biomarker.

Reference	*n* in group (irreversibly inflamed/ non-inflamed)	Biomarker	Function	Target	Analyte	Analysis	Significant difference between groups
Cytokines							
Nakanishi et al. 1995 [44]	27/9	IL-1α	Regulates immune and inflammatory reactions; stimulates bone resorption	Protein	PB	ELISA	n
		IL-1β	Regulates immune and inflammatory reactions; stimulates bone resorption	Protein	PB	ELISA	n
Elsalhy et al. 2013 [45]	43/25	IL-2	Regulates the activities of leukocytes	Protein	PB	ELISA	n
Nakanishi et al. 1995 [44]	27/9	IL-6	Regulator of T- and B-cell growth, acute phase protein production	Protein	PB	ELISA	*Not detected*
Elsalhy et al. 2013 [45]	43/25			Protein	PB	ELISA	y
Karapanou et al. 2008 [1]	17/17	IL-8	Recruitment and activation of neutrophils	Protein	GCF	ELISA	y
Elsalhy et al. 2013 [45]	43/25			Protein	PB	ELISA	y
Elsalhy et al. 2013 [45]	43/25	IL-10	Multiple effects in immunoregulation and inflammation; anti-inflammatory	Protein	PB	ELISA	y
Nakanishi et al. 1995 [44]	27/9	TNF-α	Delays neutrophil apoptosis	Protein	PB	ELISA	n
Karapanou et al. 2008 [1]	25/25			Protein	GCF	ELISA	*Not detected*
Elsalhy et al. 2013 [45]	43/25			Protein	PB	ELISA	y
Elsalhy et al. 2013 [45]	43/25	IFN-γ	Cytokine that is critical for innate and adaptive immunity	Protein	PB	ELISA	y
Proteases and other enzymes							
Zehnder et al. 2011 [33]	16/12	MMP-9	Hydrolysis of intercellular matrix; regulatory factor for neutrophil migration across basement membranes	Protein activity	Dentinal fluid	Enzyme assay	y
Nakanishi et al. 1995 [44]	27/9	Elastase	Cleavage of elastin, collagen, proteoglycans	Protein	PB	ELISA	y
Vasoactive agents							
Lepinski et al. 2000 [41]	11/10	Bradykinin	Vasodilator involved in pain and inflammation mechanisms	Protein	Extracellular pulpal fluid	RIA	y
Bowles et al. 2003 [42]	16/8			Protein	Extracellular pulpal fluid	RIA	y
Others							
Nakanishi et al. 1995 [44]	27/9	IgG	Antigen neutralization	Protein	PB	ELISA	y
Nakanishi et al. 1995 [44]	27/9	IgA	Antigen neutralization	Protein	PB	ELISA	y
Nakanishi et al. 1995 [44]	27/9	IgM	Antigen neutralization	Protein	PB	ELISA	y

(*Continued*)

Table 2. (*Continued*)

Reference	*n* in group (irreversibly inflamed/ non-inflamed)	Biomarker	Function	Target	Analyte	Analysis	Significant difference between groups
Nakanishi et al. 1995 [44]	27/9	PGE2	Multiple pro-inflammatory and immunomodulatory effects	Protein	PB	ELISA	y
Evcil et al. 2006 [46]	16/16	Serum NO	Cellular signaling molecule involved in many physiological and pathological processes	Protein activity	Peripheral blood serum	Serum assay	n

y: Yes; n: No.

Analytes were mostly either pulpal blood (PB) or gingival crevicular fluid (GCF). Extracellular pulpal fluid and peripheral serum were used in one study each. Analytical methods used included radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and specific serum or enzyme assays.

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Under normal conditions, very few immune cells are present in the dental pulp [101]. In the presence of infection (i.e. caries), immune cells are recruited to the pulp even in the absence of direct bacterial contact on the pulp tissue itself. The permeability of dentin to soluble bacterial products allows pulpal response to occur prior to carious pulpal exposure. These soluble bacterial products, along with components of the complement system and products of the lipoxygenase pathway of arachidonic acid metabolism are chemotactic for leukocytes [102].

The exponential increase in the number of infiltrating leukocytes brings with it a corresponding increase in lysosomal enzymes that cause tissue damage. Proteases like elastase and MMPs (Tables 1 and 2) cleave elastin and proteoglycans that destroy the pulp tissue resulting in irreversible damage [33, 58, 63]. Furthermore, the accompanying spike in inflammatory mediators like PGE2, cAMP, COX-2, CGRP, neurokinins and others stimulate vasodilation and microvascular permeability by binding into their respective receptors (i.e. EP2/3 receptor for PGE2) and induce cytoskeletal rearrangement or contraction of vascular smooth muscle [103].

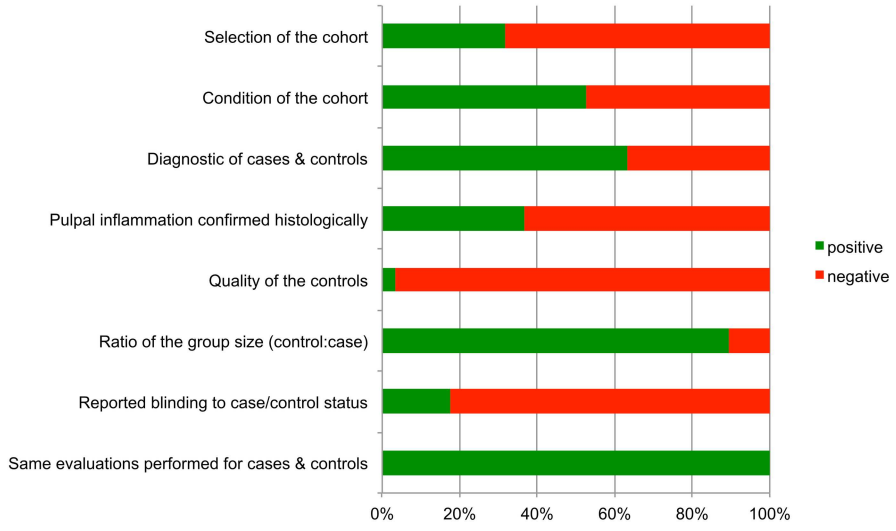

Fig 3. Bar chart showing the quality ratings of the included studies based on a modified Newcastle-Ottawa-Scale.

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Equally as important is the action of neuropeptides (e.g. substance P, calcitonin-gene related peptide) (Table 1). These neuropeptides typically reside in endings of afferent nerve close to blood vessels but also associated with macrophages and odontoblasts [104]. As a response to stimuli, afferent nerve sprouting has been demonstrated, and with it an increase in neuropeptide concentration [105], which can cause spontaneous pain, allodynia or hyperalgesia in teeth with pulpitis.

Simultaneous to the destructive effects of leukocytic infiltration is the capability of these cells to induce repair through the release of VEGF, TGF-B, GM-CSF and others (Tables 1 and 2) that induce alterations of the local extracellular matrix, promote induction of endothelial cells to migrate or proliferate, and inhibition of vascular growth with formation of differentiated capillaries [106]. The increased expression in inflamed pulp of toll-mediated human beta-defensins (hBD) [50] that play an important role in the innate host defense against bacterial invasion, contribute to promotion of adaptive immune responses, and show chemotactic activities further underscore the dynamic range of response of the dental pulp during inflammation. In addition, it can also be appreciated that during pulpal inflammation, the anti-inflammatory effects of various mediators such as tissue inhibitors of matrix proteinases (TIMPs), siRNA [94, 107] and others also come into play.

As a direct result of the release of inflammatory biomarkers, pulpal responses include classical signs of inflammation specifically a vascular response, along with changes in mediator profiles and cellular constituents. The transition from reversible to irreversible pulpitis has been broadly characterized by a migration of dendritic cells towards odontoblasts and accumulation of immune cells [108]. However, a more detailed analysis such in the majority of studies included in this paper evaluating biomarkers of pulpal inflammation demonstrates (statistical) significant differences between a clinically diagnosed healthy or irreversibly inflamed pulp at the molecular level.

Moreover, the analytes were obtained via different approaches both from the pulp directly as well as indirectly from tissue fluid. Fig 4 illustrates the potential sampling sites for molecular pulpal diagnostics [34]. While having the benefit to show a direct picture of intrapulpal conditions using pulpal blood [26] or whole pulp tissue requires access to the pulp space and is

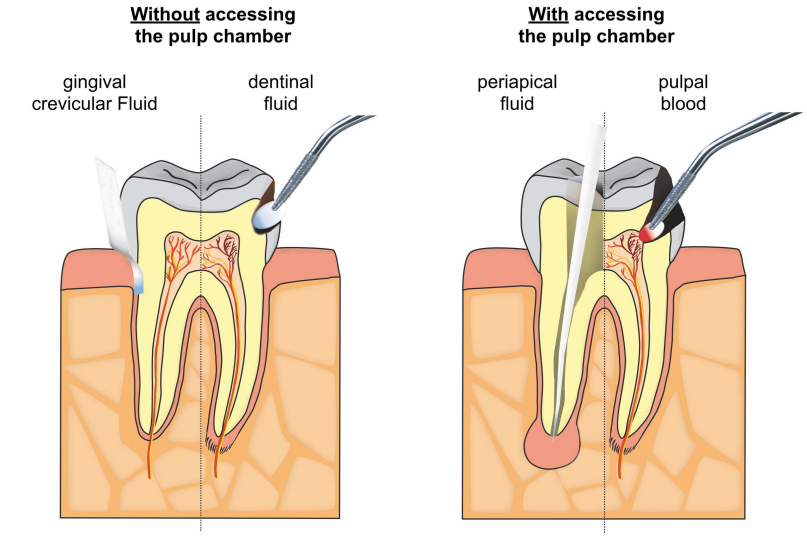

Fig 4. Actual and potential sampling sites to assay pulpal mediators.

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therefore not applicable as a chairside screening tool. Conversely, indirect methods such as dentin fluid collection or assessment of mediators in GCF can be performed clinical in a less invasive way. Dentin fluid is the extracellular fluid that is contained within dentinal tubules [109]; its composition includes inflammatory mediators and vasoactive compounds associated with inflammation. While initial evidence suggested that these mediators can be assayed [33] problems exist with protein yield [27] and the need to remove the existing restoration or in other cases to prepare an initial cavity deep in dentin.

GCF was used to sample mediators in one study included here (Table 2) [1]. This fluid is an exudate that from the gingival crevice and it contains several host factors including antibodies, bacterial antigens, proteins, and cytokines [110, 111]. GCF analyses may be promising due to the ease of application. Moreover, it may be possible to assess the dynamics of apical periodontitis using GCF [34]. However, the major drawback in the evaluation of host mediators in GCF is that tissue inflammation, independent of its cause, is a non-specific process of innate immunity [112] and this makes it difficult to distinguish on a molecular level between a marginal and apical periodontal inflammation. When sampling from the GCF for pulpal diagnostics, this potential drawback could be overcome by (i) creating healthy periodontal conditions, (ii) averaging out several sites on one or multiple teeth, (iii) combining clinical and radiographic observations, or (iv) defining a specific pattern of metabolites relevant to the pulp and not the periodontium, or possibly by other as yet unidentified means. Furthermore, the detection of mediators of pulpitis in GCF is impacted by the need for these compounds to reach the periodontal ligament and ultimately the gingival crevice in sufficient concentrations. Indeed, the notion that mediators will diffuse from the pulp via dentinal tubules or accessory canals to the periodontal ligament has been called into question [87]. Periapical fluid samples, while requiring a direct access to the apical site, are of interest to determine the level of systemic inflammation [28].

Discovering an improved method to determine the present inflammatory condition of the pulp could be of great value: on the one hand, pulp necrosis is one of the most frequent complications after coronal restoration of assumed non-inflamed (vital) teeth, on the other performing a full pulpectomy on teeth that could have been kept vital (at least in part) suggests that overtreatment may occur in many cases [113]. Endodontic diagnosis should therefore focus on either the extent of the microbial infection or the inflammatory reaction of the host tissue; however, current methods do neither [14, 23, 34].

Keeping a pulp vital offers distinct advantages compared to root canal treatment: the protective immune capacity of the pulp remains preserved and the remaining tooth structure gets not unnecessarily weakened by access cavity preparation and root canal enlargement. Unfortunately, the only available long-term outcome studies on direct pulp capping procedures (i.e. direct pulpal interventions), which attempt to maintain pulpal vitality, show unsatisfactory success rates as low as 20% after ten years [114]. The development of biocompatible materials facilitates a wound closure free of inflammation after pulpal capping procedures or partial pulpotomy [108]. However, the likelihood of a pulp to survive such procedures remains questionable using current schemes for assessment of pulpal inflammation.

One limitation of this systematic review is that merely 2 out of 57 studies [33, 45] were specifically designed to investigate potential biomarkers in the context of pulpal diagnostics. Most of the studies analyzed here merely target the presence of molecules and their function in pulpal inflammation. Nevertheless, based on the current state of knowledge this review provides an overview on molecules that are present and measurable during pulpal inflammation and therefore potentially can serve as a biomarker for pulpal inflammation. This may provide impulses for further research. This research needs to explore patient (age, gender, systemic condition) and infection related factors (varying composition of the microbiological

infection). Clinical investigations should be conducted that are specifically designed to confirm the results collected from the research collected here. More specifically mediator profiles should be assessed in defined clinical scenarios. In addition, the assays methodology should be tested for their applicability with the possible substrates. The ultimate goal should then be to develop an inexpensive chairside test for non-invasive molecular pulp diagnostics. In fact, such a chair-side assay, based on the immunochromatographic detection of MMP-8 specific antibodies, is already commercial available to diagnose periodontal inflammation [115]. For endodontic procedures of the future, such as partial pulpotomies and pulp regeneration, a comparable test will be of significant value.

Indeed, various biomarkers that are produced by cellular components of the dental pulp can provide a snapshot of the biological mechanisms that propel this immunocompetent tissue towards healing or necrosis. The imbalance between tissue destructive molecules like proteases and tissue inductive molecules like VEGF may serve as a diagnostic or prognostic tool for endodontic intervention. The challenge remains on developing a method to make these biomarkers readily measureable in a clinical setting.

Conclusions

In the included studies, irreversible pulpitis was associated with different expression of various biomarkers compared to non-inflamed controls. These biomarkers were significantly expressed not only in pulp tissue, but also in gingival crevicular fluid that can be collected non-invasively and in dentin fluid that can be analyzed without extirpating the pulpal tissue. This may be used to accurately differentiate diseased from healthy pulp tissue. The main current challenges in the clinical application of biomarkers lie in the identification of biomarkers or biomarker subsets that reliably correlate with pulpal inflammation, the improvement of sample collection (substrate and protein yields), and their analysis (interference of the biomarkers with inflammation of other than pulpal origin). If these hurdles can be overcome, a more accurate pulpal diagnosis and more predictable vital pulp treatment regime may create better clinical outcomes.

Supporting Information

S1 Table. PRISMA Checklist.
(DOCX)

S2 Table. Example of search strategy employed for this literature review.
(DOCX)

S3 Table. Hits from the literature search obtained with the different databases.
(DOCX)

S4 Table. Studies excluded from the final analysis.
(DOCX)

S5 Table. Assessment of the study quality using the modified Newcastle Ottawa Scale.
(DOCX)

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Paper 3

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Comparison of vehicles to collect dentinal fluid for molecular analysis

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ABSTRACT

Objectives: To test the hypothesis that a material with higher water absorption than polyvinylidene fluoride (PVDF) could increase the yield of target molecules from exposed dentine.

Methods: In a series of standard tests, different cellulose membranes were compared to a PVDF counterpart for their ability to absorb water and release protein. In a subsequent randomized clinical trial, the cellulose material with the most favourable values was compared to PVDF regarding the levels of MMP-2 that could be collected from exposed dentine of healthy human teeth during filling replacement. MMP-2 levels were determined by enzyme-linked immunosorbent assay (ELISA). Data from the laboratory experiments were compared between materials using the appropriate parametric tests. The frequency of cases yielding quantifiable levels of MMP-2 was compared between materials by Fisher's exact test. The level of significance was set at 5%.

Results: The cellulose membrane with the largest pore size (12–15 μm) absorbed significantly ($P < 0.05$) more water than PVDF. It showed a protein release that was similar to that of PVDF, while the cellulose membranes with smaller pore size retained significantly more protein ($P < 0.05$). Using the large-pore cellulose membrane, MMP-2 could be collected at a quantifiable level from the dentine of healthy teeth in 9 of 13 cases, compared to 1 of 13 with the PVDF membrane ($P < 0.05$).

Conclusions: Under the current conditions, a large-pore cellulose membrane yielded more of a molecule of diagnostic value compared to a standard PVDF membrane.

Clinical significance: Molecular diagnostics of dentinal fluid are hampered by low yields. In the current study, it was shown that cellulose membranes are more useful to collect MMP-2 from dentinal fluid than PVDF membranes.

1. Introduction

A recent systematic review identified sparse evidence regarding the accuracy of current pulp tests.[1] When a tooth contains an allegedly vital pulp before it is restored, the dentist has but few means to decide whether to perform a root canal treatment prior to restoration or not. This may explain why pulp necrosis is the most common biological complication in abutment teeth.[2,3]

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A novel experimental approach to non-invasively identify the state of the pulp is molecular diagnostics.[4] It is based on the collection and analysis of dentinal fluid.[5,6] However, the collection of dentinal fluid is not unproblematic. The fluid and protein flux across the pulpodentine complex is influenced by multiple factors.[5] In addition, a simple and clinically useful tool for dentinal fluid collection is currently not available. In the first trial on the levels of matrix metalloproteinase-9 (MMP-9), an enzyme associated with pulpal breakdown,[7] a folded polyvinylidene fluoride (PVDF) filter membrane was applied to exposed dentine for dentinal fluid collection. This was based on the excellent protein release from PVDF.[8] However, the first clinical study using this approach showed that the target protein was below the detection limit in the majority of the samples, also those that should contain it. It was concluded that better vehicles for dentinal fluid collection must be identified to increase the yield of target molecules before further studies can be performed along this line.

It was the aim of the current investigation to test the hypothesis that a material with higher water absorption than PVDF could increase the yield of target molecules from exposed dentine. In standard laboratory tests, cellulose filter membranes of similar thickness and different pore size were compared to the PVDF membrane used in the first clinical trial[4] regarding their water absorption and protein release. Subsequently, the cellulose membrane with the most favourable values was compared to the PVDF counterpart in a randomized clinical trial. The outcome measure was the yield of a metalloproteinase from exposed dentine of clinically healthy teeth in need of restoration replacement. The hypothesis tested here was that cellulose membranes absorb more water than PVDF counterparts, and thus could increase the yield of diagnostically relevant molecules.

2. Materials and methods

2.1. Filter membranes under investigation

Three types of cellulose membranes were compared. They were Sartorius 1288, 1289, and 1290 (Sartorius Stedim Biotech GmbH, Göttingen, Germany). These filter membranes were available as sheets, which were required for the water absorption test (below). They had a standard thickness of 0.21 mm and a pore size of 12–15 μm (1288), 8–12 μm (1289), and 3–5 μm (1290). The PVDF membrane under investigation (Merck Millipore, Billerica, MA, USA) had a thickness of 0.125 mm and a pore size of 0.45 μm.

2.2. Water absorption

For this test, the method according to Klemm was used (DIN 53 106). In brief, the filter membranes were cut to a width of 1.5 cm and a length of 10 cm. One end of these strips was attached to a custom-made holder. For measurements, the holder was placed over a glass container filled with deionized water, so that the membrane strips were immersed 2 mm below the water surface. The height of the water rise was marked with a sharp pencil on the membranes after 30 s, 60 s, and 120 s of immersion. This test was performed three times per material.

2.3. Protein release

For this test and the subsequent trial, round filter membranes with a diameter of 8 mm were used. Using a micro precision pipette (Gilson, Middleton, UK), 5 μL of a 2 mg/mL bovine serum albumin (BSA) solution (Pierce, Rockford, USA) was blotted on the membranes. Subsequently, membranes were transferred to microcentrifuge tubes (Eppendorf, Hamburg, Germany) containing 1 mL of sterile distilled water. The tubes were vortexed for 5 s. Subsequently, each membrane was transferred to a new microcentrifuge tube and again washed in 1 mL of distilled water as described. The procedure was repeated once more for a total of three washing steps. Protein recovery was measured using a commercially available protein assay (BCA Protein Assay Kit, Pierce) against a BSA standard curve. The absorbance at 562 nm was measured using a microplate reader (Epoch, BioTek, Luzern, Switzerland). Measurements were corrected for blank reading (mere membrane without protein processed as described).

2.4. Clinical trial

This trial was approved by the institutional review board (Canton of Zürich Ethics Commission). The patients participating did so by giving informed written consent.

Sartorius 1288 filter membranes were compared to Millipore PVDF counterparts for their capacity to collect a metalloproteinase from exposed dentine of clinically healthy teeth in need of filling replacement. MMP-2 was chosen as the target molecule, because this enzyme is involved in normal tissue turnover and thus present in healthy pulp tissue.[7] The membranes were folded to form a cone as described.[4] They were kept in micro centrifuge tubes with low protein binding (Life Technologies, Carlsbad, CA, USA). The membranes and tubes were sterilized in ethylene oxide (3M Sterigas, Two Harbors, MN, USA).

Thirteen lateral teeth from 8 patients (6 males and 2 females, aged 29–56) were selected for this study. These teeth reacted normal to cold test, and showed no signs of caries or inflammation. They required replacement of a filling for prosthetic or aesthetic reasons. Teeth were treated under local anaesthesia and rubber dam isolation. The occlusal aspect of the filling reaching into the dentine was removed first, i.e. the inter-proximal aspects of the fillings were left in place, to exclude contamination with crevicular fluid. Diamond-coated burs were used in a high-speed hand piece under water-cooling to remove the old filling. When the dentine was exposed, a coin was flipped to determine which material to use first. The cavity was dried with compressed air. Thereafter, the first folded membrane was applied to the exposed dentine using sterile cotton pliers (Fig. 1). The membrane was kept in place for 30 s. Subsequently, and without any intermediate procedure, the second membrane of the alternative material (as defined by the randomization process) was applied for 30 s. Membranes were immediately transferred to their microcentrifuge tubes, which were kept on ice. These tubes were then

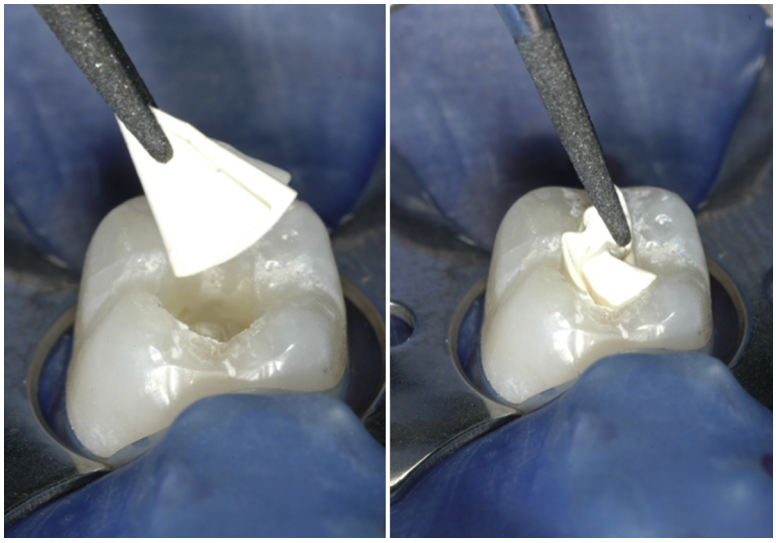

Fig. 1 – Collection of dentinal fluid from exposed dentin using a folded PVDF membrane. Left panel reproduced with premission from an earlier publication (4).

transferred to a freezer and kept at −20 °C until further analysis.

On the day of analysis, the samples were eluted in 330 μL of sterile phosphate buffered saline (PBS, pH 7.2) by centrifuging at 2000 × *g* for 30 min at 4 °C. The levels of MMP-2 in these samples were measured by a commercially available specific enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's instructions (Duoset, R&D Systems, Abingdon, UK). The absorbance at 450 nm was assessed using a microplate reader (Epoch), with a wavelength correction set at 570 nm to subtract background. A standard curve was generated using a four-parameter logistic curve fit for each set of samples assayed. The concentrations of MMP-2 were expressed as ng/mL/30 s of collection. The sensitivity of this assay, defined as the lowest detectable protein concentration that could be differentiated from zero, was 1.25 ng/mL.

2.5. *Statistics*

Values relating to water absorption were analysed using two-way repeated measures ANOVA to consider the impact of time and membrane type on water rise. Water rise at each time point and protein release were compared between materials using one-way ANOVA followed by Tukey's HSD test. The frequency of detection of MMP-2 in the clinical trial between materials was compared using Fisher's exact test. MMP-2 data from the clinical trial was skewed (Shapiro–Wilk test) and thus compared between groups using Wilcoxon Signed Rank test. The alpha-type error was set at 5% ($P < 0.05$).

3. Results

In the analysis of the values generated in the water absorption test according to Klemm, two-way ANOVA showed that both time and membrane material had a significant ($P < 0.05$) impact on water rise. Cellulose membranes took up water faster and in larger quantity than the PVDF material under investigation (Table 1). The cellulose materials with the larger pore size showed higher water absorption than the filter membrane with the small pore size.

Protein release was statistically similar ($P > 0.05$) between the cellulose materials with the large pore size (Sartorius 1288 and 1289) and the PVDF membrane. After 3 wash steps, 1288 released 93 ± 9% of the BSA, 1289 released 96 ± 3%, and the PVDF membrane 101 ± 4%. The cellulose material with the small pore size (1290) released significantly ($P < 0.05$) less protein, 81 ± 4%.

In the clinical trial, the cellulose material that performed most favourably in the laboratory screening (1288) was compared to the PVDF filter membrane regarding the detection of MMP-2 from exposed dentine of clinically healthy teeth with vital pulps. Round membranes of 8 mm diameter were folded to form tapered cones. The cone was pressed against the exposed dentine using cotton pliers. The materials were applied to the exposed dentine in random sequence during the trial, 1 membrane per material and tooth. With cellulose, MMP-2 could be detected in 9 of 13 cases, compared to 1 of 13 with PVDF ($P < 0.05$). Consequently, the levels of MMP-2 were

Table 1 – Height of water rise at different times in mm (Klemm method).

Membrane	30 s	60 s	120 s
Sartorius 1288	21.7 ± 1.2^{A}	31.0 ± 1.0^{A}	37.7 ± 0.6^{A}
Sartorius 1289	20.0 ± 1.0^{A}	31.0 ± 4.4^{A}	37.7 ± 0.6^{A}
Sartorius 1290	14.0 ± 1.7^{B}	17.7 ± 2.1^{B}	20.0 ± 0.0^{B}
Millipore PVDF	9.0 ± 0.0^{C}	13.0 ± 0.0^{C}	20.0 ± 0.0^{B}

Values indicate means and standard deviations of triplicates. Two-way repeated measures ANOVA showed that both time and membrane had a significant ($P < 0.05$) impact on water rise. Data sets sharing a superscript letter did not differ significantly at a given point in time (one-way ANOVA, Tukey's HSD).

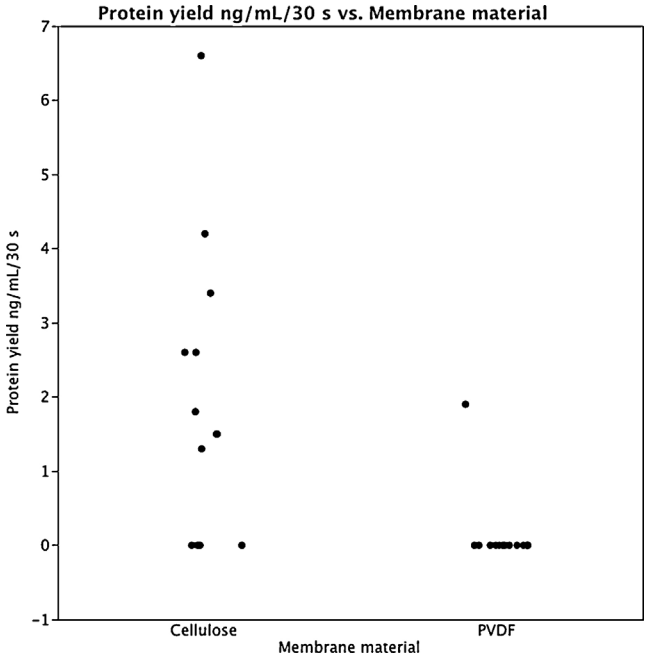

Fig. 2 – Dot plot depicting the MMP-2 yields from the two different filter membranes under investigation. Materials were applied to exposed dentin for 30 s. There was a significant difference in MMP-2 yields between the two materials under investigation (Wilcoxon Signed Rank test, P = 0.0023).

significantly higher ($P < 0.05$) when a cellulose membrane was used to collect dentinal fluid (Fig. 2). MMP-levels were statistically similar ($P > 0.05$) between first and second collections, indicating no influence of whether a membrane material was used first or second on a specific tooth (Fig. 3).

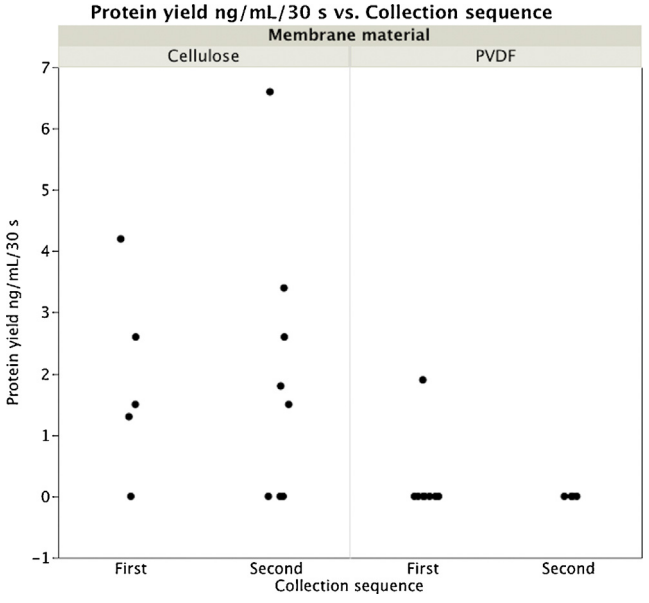

Fig. 3 – Dot plot relating MMP-2 yields to the sequence the filter membranes were applied in the clinical trial. There was no difference in yield whether a membrane was applied first or second (Wilcoxon Signed Rank test, P > 0.05).

4. Discussion

The current study showed that cellulose of large pore size showed clear advantages over PVDF in dentinal fluid collection. This was attributed to the better water absorption of cellulose compared to PVDF.

The clinical part of this study was limited by the fact that only clinically healthy teeth were included. This was done to assess the possibility to consistently collect steady-state molecules present in pulpal interstitial fluid. While the cellulose membrane under investigation performed clearly better than the PVDF membrane that was used in a first trial,[4] the target molecule was still not detected in 30% of the cases. This could be related to differences in dentine microstructure between the teeth under investigation.[9] It may be so that dentinal fluid collection is simply not possible in all teeth, especially those with complete sclerosis of dentine.[10] However, the repeated collection with the 2 different materials yielded similar amounts of the target molecule (Fig. 3). This would suggest that in future trials, at least 2 sampling procedures should be performed per tooth in order to increase fluid yield. Alternatively, the membrane used for collection could also be left in place for longer periods of time. As suggested by the in vitro part of the current communication, exposing a filter membrane to water for 1 min instead of 30 s may lead to better water uptake. A further limitation of this study was the low number of teeth under investigation. However, we merely compared 2 methods of dentinal fluid collection in healthy teeth. Based on the in vitro results, a difference between the 2 methods of more than 50% in absolute MMP-2 levels was expected. As indicated by the highly significant difference between the 2 methods, this assumption was correct, and the number of samples was adequate.

Absorbency of the vehicles used for dentinal fluid collection may play a role because the natural flow of fluid through dentine is rather low.[5] It may be so that the cellulose membrane actively soaked more fluid from the dentine wound than the PVDF membrane did. Dentinal fluid flow is related to pulpal pressure.[11] This pressure might vary based on the inflammatory state of the underlying pulp.[12] However, even necrotic teeth contain dentinal fluid.[13] Using the cellulose membrane technique described here, we were able to collect MMPs from teeth with necrotic teeth also (data not shown). Consequently, absorption of fluid through dentine by cellulose may be necessary to get maximum yields regardless of the condition of the pulp. This, however, needs to be investigated in future trials.

In the current study, a simple ELISA assay was used. In the first trial on MMP-9 levels in the dentinal fluid of healthy and inflamed pulps, a more sensitive, yet also more complex assay was employed, which detects the target molecule indirectly via substrate turnover.[4] That assay detected MMP-9, a molecule related to pulpal tissue breakdown, in the dentinal fluid of 7 out of 16 teeth diagnosed with irreversible pulpitis, compared to 0 of the 12 healthy control teeth. That first study should now be repeated to investigate whether the use of a cellulose material for dentinal fluid collection would allow for sample analysis with straightforward ELISA kits, or whether

more sensitive assays are still required. However, even though the kit used here is easy to use, the current approach to analyse dentinal fluid would still be far away from chair-side practicability in a dental office setting.

As the field of molecular diagnostics of the pulp is relatively new, basic studies such as the current investigation are necessary to identify ideal and simple tools for the collection of dentinal fluid. Dentinal fluid could, for a long time, only be collected from extracted teeth, e.g. by heating the dentine or by centrifugation.[14,15] In the original study on dentinal fluid analysis of vital human teeth, phosphate-buffered saline was placed in the cavity over the exposed dentine for 15 min and then collected using micropipettes.[6] Similar approaches were also used to study dentinal fluid flux in dog teeth.[5,11] These collection methods, however, are impracticable in the clinical situation. The current research aimed towards the development a chair-side assay that could reflect the health of pulp before a tooth is restored. Whether this goal can ever be achieved is not yet clear. Nevertheless, current knowledge on pulp biology and sensitive molecular assays could prove to be useful in this context. It is beyond doubt that pulpal breakdown is triggered by bacterial invasion of dentine,[16] yet propagated by enzymes secreted by polymorphonuclear leukocytes (PMNs).[17] The idea to use PMN-associated enzymes as markers for pulpal health was formulated, yet not tested, more than 20 years ago.[5] In this context, the MMP system of enzymes is interesting. Some of these enzymes are produced by resident cells.[18] They are related to normal tissue turnover, and are thus not elevated or even less active in inflamed pulps compared to normal counterparts.[7] On the other hand, PMN-related enzymes such as MMP-9 (also called neutrophil gelatinase), are strongly correlated to pulpal inflammation and breakdown.[7] It may thus be suggested to use the ratio between MMP-9 and MMP-2 in future clinical trials to relate dentinal fluid composition to the state of the pulp. It must be cautioned, however, that MMPs are also contained in the dentine itself, and can be released by caries-related acids.[19,20] Furthermore, proteins of varying size and structure might be retained differently during their passage through the pulp–dentine complex.[5] It may thus be wise to investigate the levels of different proteins and their relation to different conditions of the pulp to identify the most robust markers. In summary, much work lays ahead to further develop dentinal fluid-based diagnostics.

5. Conclusion

The identification and development of better vehicles for dentinal fluid analysis is essential for the propagation of non-invasive molecular diagnostics of the dental pulp. This research can be seen as a first step into that direction. Future clinical trials should now reveal whether a cellulose membrane is a suitable collection tool to consistently identify marker molecules related to pulpal health and disease.

Conflict of interest

The authors declare that they have no conflict of interest

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Paper 4

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Periapical fluid RANKL and IL-8 are differentially regulated in pulpitis and apical periodontitis

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ABSTRACT

The dental pulp space can become infected due to a breach in the surrounding hard tissues. This leads to inflammation of the pulp (pulpitis), soft tissue breakdown, and finally to bone loss around the root apex (apical periodontitis). The succession of the molecular events leading to apical periodontitis is currently not known. The main inflammatory mediator associated with neutrophil chemotaxis is interleukin-8 (IL-8), and with bone resorption the dyad of receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG). The levels of RANKL, OPG and IL-8 were studied in periapical tissue fluid of human teeth ($n = 48$) diagnosed with symptomatic irreversible pulpitis (SIP) and asymptomatic apical periodontitis (AAP). SIP represents the starting point, and AAP an established steady state of the disease. Periapical tissue fluid samples were collected using paper points and then evaluated using enzyme-linked immunosorbent assays (ELISAs). Target protein levels per case were calibrated against the corresponding total protein content, as determined fluorometrically. RANKL was expressed at significantly higher levels in SIP compared to AAP ($P < 0.05$), whereas OPG was under the detection limit in most samples. In contrast, IL-8 levels were significantly lower in SIP compared to AAP ($P < 0.05$). Spearman's correlation analysis between RANKL and IL-8 revealed a significantly ($P < 0.05$) negative correlation between the two measures (rho = −.44). The results of this study suggest that, in the development of apical periodontitis, periapical bone resorption signaling, as determined by RANKL, occurs prior to inflammatory cell recruitment signaling, as determined by IL-8.

1. Introduction

The oral cavity is a unique environment, where hard tissue structures (teeth) breach a soft tissue barrier into a space replete with microorganisms. Consequently, when the microorganisms manage to overcome this barrier, an opportunistic infection can occur. Examples of opportunistic oral infections include caries, periodontitis, and apical periodontitis [1]. Apical periodontitis is fairly common in adults, with roughly one in three individuals affected [2]. As any other opportunistic infection, apical periodontitis is characterized by a complex interplay between microbial tissue invasion and host defense [3]. In the course of pulpal inflammation, the soft tissue inside the root canal system is digested by proteolytic enzymes, which are produced by neutrophils [4,5]. Unless the entryway for the microorganisms into the pulp space is blocked by intervention [6], the whole canal system can gradually become infected, and an inflammatory lesion establishes around the apical region to keep this infection under control [7]. One histo-pathologic endpoint of periapical inflammation is bone loss, which may occur to increase vascularization at the portals of the apex, thus blocking the infection in the root canal space from affecting the host [7,8].

The primary line of defense against microorganisms is the innate immune system. Pulpal and later periapical inflammatory responses are characterized by the influx of neutrophils [7]. The main molecules associated with neutrophil recruitment and activation to the site of infection is interleukin-8 (IL-8, CXCL8) [3]. IL-8 is produced at local sites of inflammation and has been associated with pulpal breakdown and apical periodontitis [9,10]. Periapical bone loss is a hallmark of apical periodontitis. On the molecular level, bone resorption is orchestrated by the interplay of receptor activator of NF-κB ligand (RANKL) and osteoprotegerin

Abbreviations: IL, interleukin; RANKL, receptor activator of NF-κB ligand; OPG, osteoprotegerin; SIP, symptomatic irreversible pulpitis; AAP, asymptomatic apical periodontitis.

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(OPG). The presence of RANKL activates its cognate RANK receptor and stimulates differentiation of precursor cells into osteoclasts. On the contrary, the OPG soluble decoy receptor blocks RANKL, hence preventing its interaction with RANK, and therefore inhibits osteoclast activation and subsequently bone resorption [11,12]. The RANKL–OPG system is clearly involved in the pathogenesis of periodontal disease, and its relative RANKL/OPG ratio can indicate the occurrence, yet not the progression, of the disease [13]. Since apical periodontitis is also characterized by bone loss, it is fair to postulate a role of this bi-molecular system in the pathogenesis of this disease as well. Indeed, a recent comprehensive review has summarized the information available of the RANKL–OPG system in the context of pulpal and periapical disease, identifying a lack of conclusive information [14].

What is currently not known in the context of periapical inflammation and bone resorption, is the succession of the molecular events that culminate in bone resorption or inflammatory cell recruitment. It is hypothesized that the molecular activation of bone resorption in the periapical region, hence the regulation of RANKL and OPG, should precede the chemotactic signals that lead to inflammatory cell recruitment, as evaluated by the regulation of IL-8. Therefore, it was the aim of the current study to investigate the periapical tissue fluid levels of RANKL, OPG and IL-8 in teeth with inflammation restricted to the pulp space, and compare these to counterparts from teeth with an established periapical inflammatory lesion.

2. Materials and methods

2.1. Patients, operative procedures and periapical fluid collection

This study was approved by the Ethics Review Board of the Canton of Zürich (KEK-ZH-No. 2011-0253/4) and was conducted in accordance with the guidelines of the World Medical Association Declaration of Helsinki. It is confirmed that this cross-sectional study conforms to STROBE guidelines for observational studies. All patients were treated at the Department of Preventive Dentistry, Periodontology and Cariology, University of Zürich, Center of Dental Medicine, Switzerland by one operator specialized in endodontics (DKR). The participants were in need of a root canal treatment. They were either referred for the treatment or attended the department's emergency unit during service hours. The patients were asked to participate in the study when they were of full age ($\geqslant$18 y) and were excluded from the study if they: (i) refused to participate in the study, (ii) were under long-term anti-inflammatory medication, immunosuppressive chemotherapy or any antibiotic medication, (iii) were not systemically healthy (i.e. suffer from cardiovascular and respiratory disease, diabetes mellitus, HIV infection or hepatitis), or (iv) were pregnant or in lactation. The clinical condition was diagnosed according to the patient's case history, clinical inspection, palpation, tenderness to percussion, vitality testing, probing depth and radiographic examination (single-tooth radiograph, Digora, Soredex, Tuusula, Finland). The clinical conditions included in the study were symptomatic irreversible pulpitis (SIP) with normal apical tissues and pulp necrotic or previously treated teeth with asymptomatic apical periodontitis (AAP) as defined by the American Association of Endodontists [15]. The patients who entered the study gave written informed consent. All operative procedures were performed using a dental microscope under suitable magnification. For root canal treatment the patient's teeth were anesthetized with 4% articain (Septanest, Septodont, Saint-Maur-des-Fossés Cedex, France) and isolated with rubber dam. The work field was wiped with a cotton pellet soaked with 3% sodium hypochlorite (Kantonsapotheke, Zürich, Switzerland) for surface disinfection. When caries was present, it was excavated with round burs and a pre-endodontic build-up placed if deemed appropriate. The endodontic access was prepared with a sterile diamond-coated bur and the root canals were instrumented using ProTaper instruments (Maillefer Dentsply, Ballaigues, Switzerland) according to the manufacturer's recommendations. Endodontic working length was determined endometrically (Root ZX mini, J Morita Corp., Tustin, CA, USA) with a hand file (Maillefer Dentsply). The diagnosed clinical condition (SIP or AAP) was confirmed during instrumentation by assessing the content of the root canal for the presence or absence of vital tissue. The root canals were instrumented up to their apical constriction, under continuous manual irrigation with 1% sodium hypochlorite (Kantonsapotheke) using a syringe with a side-vented needle (Hawe Irrigation Probe, gauge no. 30, Kerr Hawe, Bioggio, Switzerland) one millimeter shorter than the predetermined working length. A size-15 hand file was frequently used to keep the apical foramen patent. After instrumentation to ProTaper F2, five mL of sterile physiological saline solution (.9%, B. Braun Medical AG, Sempach, Switzerland) were administered to full working length to inactivate possible remnants of NaOCl. After drying the root canal with sterile paper points a fine paper point (Orbis Dental, Münster, Germany) was inserted approximately 2 mm above the apical foramen to collect the periapical tissue fluid [16]. The paper point was kept in that position for 30 s and was then transferred into a sterile micro-centrifugation tube (Biopure 1.5 ml, Vaudaux-Eppendorf AG, Schönenbuch, Switzerland). Three paper points were collected from one canal and immediately after frozen at −80 °C until further processing. Only one root canal per tooth/patient has been sampled as described above. In case of multi-rooted teeth diagnosed with AAP one root corresponding with the radiographic lesion was sampled whereas in cases of multi-rooted teeth diagnosed with SIP the sampled canal was chosen randomly. After the periapical fluid collection the root canal treatments were finished *lege artis*.

2.2. Sample preparation

For the laboratory analysis, a protease inhibitor (complete mini EDTA free, Roche, Basel, Switzerland) was added to sterile phosphate buffered saline (PBS, pH 7.2) and the samples were re-eluted in 300 µl of this mixture. The micro-centrifugation tubes were placed for 5 h on a platform shaker at (2000 rpm) at 4 °C. Afterwards, the tubes were vortexed for 30 s and thereafter centrifuged for 10 min at 5000 rpm. The supernatant was collected and transferred into new micro-centrifugation tubes before being processed on 96-well plates for subsequent enzyme-linked immunosorbent assay (ELISA) analyses, while the resulting pellet was stored.

2.3. ELISA assays for RANKL, OPG and IL-8

The total amount of RANKL and OPG in the periapical supernatant was determined by human-specific ELISA according to the manufacturer's instructions (total sRANKL ELISA and Osteoprotegerin ELISA kits, Immundiagnostik AG, Bensheim, Germany). The concentrations of IL-8 in the supernatant were evaluated using a commercially available ELISA kit (DY208, Duo Set Human CXCL8/IL-8 ELISA, R&D Systems, Abingdon, UK). Absorbance was measured at 450 nm using a microplate reader (Epoch, BioTek, Lucerne, Switzerland), with wavelength correction of 620 nm for RANKL and OPG, and 570 nm for IL-8. A standard curve was created by a four-parameter logistic (4-PL) equation, using known concentrations of the rhRANKL, rhOPG and rhIL-8 standards, all provided in the corresponding ELISA kits. The concentrations of RANKL, OPG and IL-8 in all periapical fluid supernatants were calculated against these standard curves. Additionally, the concentrations of these analytes were also calibrated against the total protein content in each sample. Total protein in

the periapical supernatant was quantified using a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA), as recommended by the manufacturer.

2.4. Statistics and data presentation

To compare differences between clinical diagnosis groups regarding categorical variables (gender, tooth type, or jaw), the Chi-Square test was used. Normality of the data related to continuous variables was checked by the Shapiro–Wilk test. These data sets were skewed, and therefore for comparison between groups, Mann–Whitney *U* test was used. Correlation between RANKL and IL-8 levels was evaluated by the Spearman's correlation analysis. Statistically significant difference was considered at the level of $P < 0.05$. Continuous variables are presented as medians and interquartile ranges (IQRs).

3. Results

3.1. Patient demographics and samples evaluated

Fifty-eight patients ($n = 58$) were recruited between September 2011 and May 2013. Periapical tissue fluid samples were collected from all patients, as described above. Ten samples could not be further analyzed for the following reasons: (i) problems with the sampling procedure (i.e. severe blood contamination or it was impossible to sufficiently reach the periapical area with the paper point, $n = 7$), (ii) association with pus (i.e. pus discharge from the root canal, $n = 3$). The remaining 48 samples were distributed SIP ($n = 21$) and AAP ($n = 27$) among the different clinical diagnosis. The ratio between female and male patients was 29/19 and the patient's age ranged between 18.0 and 80.5 years. Twenty-eight of the samples included were from molar teeth, 12 from premolars and 8 collected from anterior teeth. Twenty-six teeth were located in the maxilla and the remaining 22 teeth in the mandible. With regards to categorical variables, there were no statistically significant differences between the diagnostic groups when compared regarding gender, tooth type, or tooth location ($P > 0.05$). Age of patients was also similar between groups, with no significant difference ($P > 0.05$).

3.2. Determination of RANKL, OPG and IL-8 levels in periapical fluid

The total protein concentration was first investigated, in order to evaluate if there were any quantitative differences in sampling amounts between the diagnostic groups. These were (median, IQR) 166 (154, 223) mg/ml in SIP and 182 (154, 257) mg/ml in AAP, and the difference proved to be statistically non-significant (Fig. 1A, $P > 0.05$). The levels of RANKL calibrated to total protein were further investigated. RANKL was detected in 20 of 21 samples in SIP (95%) and in 24 of 27 samples with AAP (88%). The median RANKL level for SIP was 1456 pg/mg total protein (IQR: 774–2567 pg/mg total protein). This was significantly ($P < 0.05$) higher in SIP than in AAP with 829 pg/mg total protein (IQR: 263 to 1785 pg/mg total protein; Fig. 1B). On the other hand, OPG was barely detected in any of the periapical fluid samples, namely in 5 of 21 samples with SIP (24%), and 3 of 27 samples with AAP (11%). The low frequency of OPG detection prohibited any meaningful statistical analysis to determine differences between both groups. Moreover, due to the same reason, it was not possible to deduce the relative RANKL/OPG ratio in these samples.

The levels of IL-8 in the periapical fluid samples, calibrated to total protein, were investigated next. IL-8 was detected in 9 of 21 samples with SIP (43%) and 20 of 27 samples with AAP (74%). Periapical fluid samples of teeth with SIP exhibited significantly ($P < 0.05$) lower levels of IL-8 (median = not detected, IQR: not detected – 23 pg/mg protein) than in AAP (median = 106, IQR: not detected – 1340 pg/mg total protein; Fig. 1C). Spearman's correlation analysis between the levels of RANKL and IL-8 in both conditions (values normalized to total protein) revealed a significantly ($P < 0.05$) negative correlation between the two measures (rho = −.44).

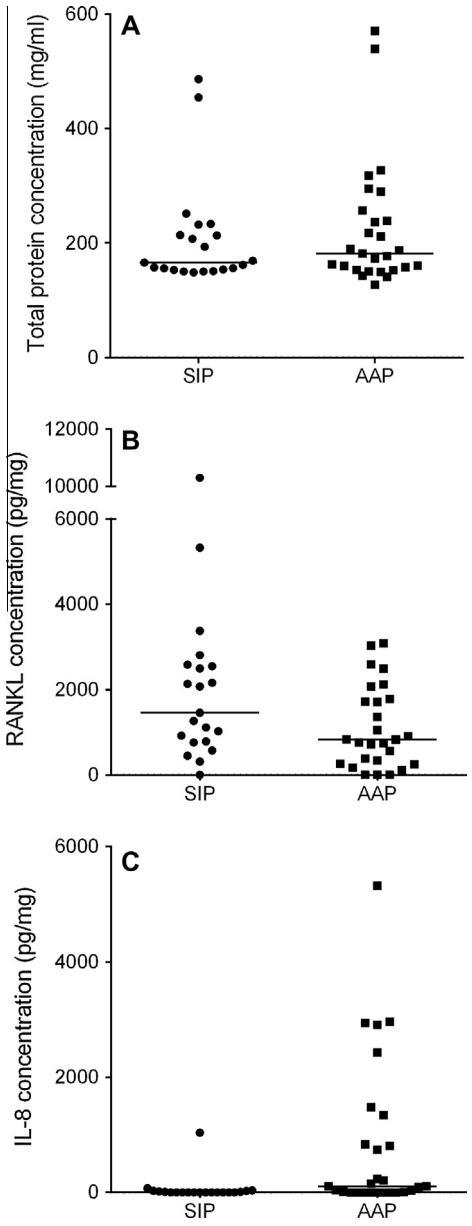

Fig. 1. Quantitative expression of total protein (A), RANKL (B) and IL-8 (C) in periapical tissue fluid samples of teeth diagnosed with symptomatic irreversible pulpitis (SIP) and asymptomatic apical periodontitis (AAP). Total protein concentration (mg/ml) was determined fluorometrically (Qubit 2.0). Samples from SIP and AAP proved to be statistically similar ($P > 0.05$). RANKL concentration was determined by human-specific ELISA calibrated against the total protein content of each individual sample (pg/mg). RANKL levels were significantly higher in samples from SIP when compared to AAP ($P < 0.05$). IL-8 concentration was determined by human-specific ELISA calibrated against the total protein content of each individual sample (pg/mg). Periapical fluid samples of teeth with SIP exhibited significantly ($P < 0.05$) lower levels of IL-8 than AAP.

4. Discussion

In this study the periapical molecular inflammatory response to an opportunistic microbial infection was investigated. The findings

demonstrate that at the starting point of apical periodontitis, as represented in SIP, where the inflammation is still confined to the root canal space, high levels of RANKL, but low levels of IL-8 are detectable in the periapical tissue fluid. In contrast, when an apical lesion is established and a chronic state of the inflammation has emerged, as is the case of AAP, significantly more IL-8, but less RANKL is present at the periapex. These findings strongly suggest, that in the kinetics of apical periodontitis, periapical bone resorption signaling, regulated by the RANKL/OPG system takes place prior to inflammatory cell recruitment mediated by IL-8, according to the hypothesis of the present study.

There are advantages employing apical periodontitis models for studying inflammatory responses such as a readily quantifiable endpoint of apical tissue destruction and the development of the pathology in a circumscribed area [8]. However, in previous studies the kinetics of apical periodontitis has been investigated mainly employing rodent models where the pulp gets exposed to the oral environment [17]. Pro-inflammatory cytokines, such as IL-1 and TNF-α, are expressed at a very early stage of the disease in rodent's periapical tissues [18]. Especially IL-1 plays a pivotal role in development and progression of apical periodontitis [8]. In addition, both IL-1 and TNF-α are potent inducers of chemokine's further downstream such as IL-8. Nevertheless, the role of IL-8 remains unclear using rodent models, particularly because one major difference between the human and murine immune system is that IL-8 is not expressed by the latter [19]. In humans, IL-8 is synthesized instantaneously at local sites of infections by a variety of tissue cells e.g. endothelial cells or fibroblasts. High molecular levels of IL-8 have been detected in inflamed human pulps and chronic apical lesions immunohistochemically [9] and in periapical exudate of teeth with symptomatic and asymptomatic apical periodontitis engaging molecular methods [20]. Collectively, these earlier observations are in agreement with the results of the present study, whereby IL-8 is detected in AAP, and at particularly higher levels, compared to the healthy apical tissue in SIP.

Another crucial biological process of apical periodontitis, namely bone resorption, was also evaluated in the periapical tissue fluid samples of this study. This was achieved by measuring the presence and levels of the RANKL–OPG system, which is of relevance to pulpal and periapical pathosis, particularly by initiating the molecular events that lead to osteoclastogenesis [14]. However, OPG could be detected in 17% of the samples. One possible explanation for this observation could be, that the major source for OPG is connective tissue [13], which is not that prominent in the tight periapical region, especially once the dental pulp has been removed, as was in the present case. Nevertheless, molecular mechanisms of bone resorption could still be assessed by the presence of RANKL in the periapical tissue fluid. RANKL was detectable in both groups and was significantly higher expressed in cases diagnosed with SIP compared to AAP. Some studies reported significantly more RANKL-positive cells [21] and RANKL mRNA expression [22] for chronic apical lesions compared to their controls. However, healthy apical periodontal ligament cells scraped of teeth extracted for orthodontic reasons frequently served as negative controls, which is not optimal for comparison with cells from the periapical region. Accordingly, a limitation of the present study was that due to ethical considerations it is not possible to collect control samples from healthy teeth. Yet, the presence of RANKL at higher levels in the apical tissue fluid from teeth with SIP compared to AAP confirmed in this study, indicates a higher regulation of bone resorption signaling events at the early, rather than the established, stages of apical periodontitis.

5. Conclusions

In conclusion, the early periapical presence of RANKL in teeth with SIP, along with the higher presence of IL-8 at the later stage of AAP, as well as the negative correlation between these two measures indicates a reverse cascade of molecular events according to the stage of the apical periodontitis. At early stages, there is an activation of bone resorption to allow the expansion of the inflammation to the periapical region. At later stages, when the bone lesion has occurred to accommodate the inflammatory infiltrate, there is instead active chemotactic signaling that may contribute to the chronicity of periapical inflammation. These molecular characteristics of periapical pathologies should be taken into consideration, as markers for monitoring the different stages of apical periodontitis, or as potential targets for adjunctive therapeutic treatment.

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Paper 5

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Identification of *Synergistetes* in endodontic infections

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ABSTRACT

The bacterial phylum *Synergistetes* consists of Gram-negative anaerobes. Oral *Synergistetes* are divided in two main clusters, namely A and B. Increasing evidence demonstrates their involvement in etiology of oral infections, including apical periodontitis. This condition causes bone loss around the apex of the tooth, subsequent to pulp inflammation (pulpitis). Although the presence of *Synergistetes* has been confirmed in endodontic infections by molecular methods, these have not been morphologically identified in the affected apical region, and their prevalence among different endodontic infections has not been determined. Therefore, the aim of this study was to evaluate the prevalence, levels and morphology of oral *Synergistetes* clusters A and B, in apical root canal samples obtained of teeth with irreversible pulpitis, pulp necrosis and apical periodontitis, or previously root-filled teeth with apical periodontitis. For their detection, fluorescence *in situ* hybridization and epifluorescence microscopy were used. *Synergistetes* cluster A was not detected in pulpitis, but was found in both apical periodontitis groups, more frequently and at higher ranges in teeth which were previously root-filled. Microscopically, they appeared as straight or slightly curved long rods. *Synergistetes* cluster B was not detected in any of the cases. *Fusobacteria* and *Actinomyces*, which are well-established taxa in endodontic infections, were detected more frequently and at higher ranges than *Synergistetes*. In conclusion, *Synergistetes* cluster A constitutes part of the mixed apical microbiota in apical periodontitis, and may be involved in its pathogenesis.

1. Introduction

Synergistetes is a recently identified bacterial phylum consisting of Gram-negative anaerobes. They are found in several microenvironments and constitute part of the human microbiota in health and disease [1–4], including the oral microbiota [5]. Phylogenetically, the oral *Synergistetes* are divided principally into clusters, namely cluster A and cluster B [2,3]. Considerable work on the role of *Synergistetes* has been made in the field of periodontal infections. They are detected more frequently and more abundantly in subgingival plaque from periodontitis-affected than healthy sites [2], or in the saliva of patients with periodontitis, compared to healthy individuals [6]. Accordingly, dental plaque from periodontitis-affected sites exhibits higher clonal abundance and diversity of *Synergistetes*, in comparison to healthy sites [7]. The presence of *Synergistetes* in dental plaque is also more pronounced in necrotizing ulcerative gingivitis, compared to plaque-induced gingivitis [8].

Widespread oral infections, such as caries, periodontitis, and apical periodontitis, are of largely opportunistic nature [9]. Apical periodontitis (AP) is the outcome of endodontic infection. It is very prevalent among adults, with an estimated one-third of the population being affected [10]. The initial steps of the disease involve the microbial invasion and inflammation of the pulpal tissue (pulpitis), primarily as a result of dental caries [11,12]. Persistent inflammation inside the root canal system causes degradation of the pulpal tissue [13] and allows for the progression of the endodontic infection. Histopathologically, this leads to the establishment of an inflammatory lesion in the bone around the apical region of the tooth, which is characteristic of AP, aiming to keep under control the recurring infection in the root canal system [14].

Synergistetes have been frequently detected at elevated numbers in root canals of teeth with chronic endodontic infections, such as AP, as identified by polymerase chain reaction (PCR)-based methods and sequencing analyses [15,16]. The size of the total

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Synergistetes population in necrotic teeth with AP is reported to range between 10^4 and 10^6 16S rRNA gene copies, while their proportion constitutes <1.0% of the total microbial community, allegedly within the detection range of other pathogens characteristic of endodontic infections [17]. Yet the differential presence of *Synergistetes* clusters A and B in the apical region of teeth with various clinical endodontic diagnoses has not been determined. Hence, the aim of this study was to evaluate the prevalence, levels and morphology of oral *Synergistetes* clusters A and B, in apical samples obtained of teeth with irreversible pulpitis but normal apical tissues, pulp necrotic teeth with AP, or previously root-filled teeth with AP. Comparatively, the presence of *Fusobacteria* and *Actinomyces* was also evaluated, as these are well-established taxa in mixed endodontic infections.

2. Materials and methods

2.1. Patients, procedures and sample collection

This study was approved by the Ethics Review Board of the Canton of Zürich (KEK-ZH-No. 2011-0253/4) and was conducted in accordance with the guidelines of the World Medical Association Declaration of Helsinki. The participating patients were in need of root canal treatment, and were treated at the Clinic for Preventive Dentistry, Periodontology and Cardiology, University of Zürich, Center of Dental Medicine, Switzerland by one operator specialized in endodontics (DKR). The patients were asked if they were willing to participate in the study if they were of full age (≥18 y), and were excluded from the study if they: (i) were unwilling to participate, (ii) were under long-term medication, such as immunosuppressive chemotherapy or any antibiotics, (iii) suffered from systemic illness (i.e. cardiovascular and respiratory disease, diabetes mellitus, HIV infection or hepatitis), or (iv) were pregnant or in lactation. The clinical condition was diagnosed according to the patient's case history, clinical inspection, palpation, tenderness to percussion, vitality testing, probing depth and single-tooth radiographic examination. The clinical conditions included in the study were irreversible pulpitis but normal apical tissues (pulpitis, $n = 27$), pulp necrotic teeth with apical periodontitis (N-AP, $n = 33$), or apical periodontitis associated with a root-filled tooth (R-AP, $n = 21$). Only one tooth per patient was included in the study. None of the sampled teeth exhibited a confirmed combined endodontic-periodontal lesion. All patients who entered the study gave written informed consent. All operative procedures were performed under a dental microscope and rubber dam isolation. The endodontic access was prepared with a sterile diamond-coated bur and the root canals were instrumented using ProTaper instruments (Maillefer Dentsply, Ballaigues, Switzerland), while endodontic working length was determined endometrically (Root ZX mini, J Morita Corp., Tustin, CA) with a hand file (Maillefer, Dentsply). The root canals were instrumented up to their apical constriction, under continuous manual irrigation with 1% NaOCl. During this process, a size-15 hand file was also used, to keep the apical foramen patent. After instrumentation to ProTaper F2, 5 ml of sterile physiological saline solution were administered to full working length to inactivate possible remnants of NaOCl. After drying the root canal with sterile paper points, a fine paper point (Orbis Dental, Münster, Germany) was inserted approximately 2 mm above the apical foramen to collect the apical tissue fluid, and the point was kept in that position for 30 s. Three consecutive paper points were collected from one canal and immediately after frozen at −80 °C until further processing.

2.2. Sample preparation

Initially, the paper point content was re-eluted in the tubes with 300 μl of sterile phosphate buffered saline (PBS), containing a protease inhibitor (complete mini EDTA free, Roche, Basel, Switzerland). The tubes were placed for 5 h on a platform shaker at (2000 rpm) at 4 °C, vortexed for 30 s, and thereafter centrifuged for 10 min at 5000 rpm. The resulting cell pellet was collected and processed for analysis, as described further.

2.3. Analysis of apical samples by fluorescent in situ hybridization (FISH) and epifluorescence microscopy

The detection and counting of bacteria in the prepared samples was performed by FISH, followed by epifluorescence microscopy, in similar principles as previously described [8]. Briefly, 50 μl of 0.9% NaCl containing RNase inhibitor (Sigma Aldrich, Buchs, Switzerland) were added onto the pelleted sample, followed by shaking for 45 min, and vortexing for 1 min. Then, 10 μl of the suspensions were mixed with 5 μl of coating buffer (0.9% NaCl, 0.02% NaN_3, 2.5×10^{-4}% hexadecyltrimethylammonium bromide) on multi-well epoxy coated Adcell slides, with a well-diameter of 4 mm (Cel-Line, Erie Sientific Company, Portsmouth, NH, USA). The slides were air-dried and fixed by a 20 min-incubation in 4% paraformaldehyde at 4 °C, washed with nanopure H_2O and then processed for FISH analysis [8]. Every well was covered with 9 μl Denhardt's Solution (diluted 1:50 in PBS), including 1:500 RNase inhibitor, to reduce non-specific probe binding to the bacterial cell wall. The slides were then incubated for 30 min at 37 °C. Four specific oligonucleotide rRNA probes were used for *Synergistetes* cluster A and *Synergistetes* cluster B bacteria [6,8], a genus-specific probe for oral *Fusobacteria* [18], and a genus-specific probe for oral *Actinomyces* [19]. The cluster classification of oral *Synergistetes* bacteria into A and B was based on earlier studies [2,6,8]. Table 1 lists the oligonucleotide sequences and Cy3 or 6-FAM labeling of the used rRNA probes (Microsynth, Balgach, Switzerland), as well as their targeted taxa. The final probe concentrations used for FISH were 5 ng/μl for Cy3 conjugates and 20 ng/μl for FAM conjugates, in the presence of 40% formamide. For hybridization, 3–4 μl of probe solution was added to the wells, and incubated for 4 h, at 46 °C. Thereafter, they were washed for 30 min, air-dried and covered with 50 μl mounting fluid and a cover-slip. An Olympus BX60 fluorescence microscope (Olympus Optical AG, Volketswil, Switzerland) was used for the quantitative evaluation of the FISH stained samples. Fluorescence and direct light images of the detected bacteria were taken by an Olympus E510 camera. The quantitative evaluation of the stained bacterial taxa was done by counting the fluorescent bacterial cells in at least 10 viewing fields per well, at 100× magnification, as previously described [20]. The lowest detection limit of the assay was 50 bacterial counts per sample.

2.4. Statistical analysis

The Chi-square test was used to compare the frequencies of detection of the different bacterial taxa among the three clinical diagnosis groups. Statistical significance level was set at $P < 0.05$.

Table 1
16S rRNA-targeted probe sequences for FISH and target taxa.

Probe	Sequence (5′–3′)	Target taxa
SYN-A1409-FAM	ACACCCGGCTCGGGTGGT	*Synergistetes* cluster A
SYN-B1149-Cy3	TCGATGGCAGTCTCGCCG	*Synergistetes* cluster B
L-ACT476-2-FAM	ATCCAGCTACCGTCAACC	Genus *Actinomyces*
FUS664-Cy3	CTTGTAGTTCCGCYTACCTC	Genus *Fusobacterium*

3. Results

The presence of *Synergistetes* in the apical samples obtained from the three different clinical conditions was evaluated using FISH and epifluorescence microscopy. The frequencies of detection of the different taxa were first calculated (Table 2). It was found that *Synergistetes* cluster A bacteria were present 10% of N-AP cases, in 24% of R-AP cases, but none of pulpitis cases. Interestingly, *Synergistetes* cluster B was not detectable in any of the groups. To confirm the presence of well-established endodontic pathogens in apical periodontitis, the *Actinomyces* and *Fusobacterium* genera were further investigated using 16S rRNA genera-specific oligonucleotide probes. It was found that *Actinomyces* were detected in 12% of N-AP cases, 48% of R-AP cases, but none of the pulpitis cases. On the other hand *Fusobacteria* were detected in 12% of N-AP cases, 38% of R-AP cases, and 11% of the pulpitis cases. These differences in detection proved to be statistically significant between clinical diagnosis groups for all taxa analyzed, with the exception of *Synergistetes* cluster B, which was undetectable under the present conditions. Regarding the numerical levels of detection of the investigated taxa, these ranged between 50 and 255 counts for *Synergistetes* cluster A, 70–1650 counts for *Actinomyces*, and 50–720 counts for *Fusobacteria* (Table 3). The highest bacterial numbers were detected in samples with clinical diagnosis of R-AP, followed by N-AP.

The investigated bacterial taxa were further characterized morphologically, as appeared by FISH staining under epifluorescence microscopy. The *Synergistetes* cluster A bacteria, detected only in the N-AP and R-AP cases, appeared as long rods, which were either in straight form (Fig. 1A, B) or curved form (Fig. 1C, D). *Fusobacteria* appeared as elongated spindle-shaped rods either as single cell bodies (Fig. 2A–D) or forming complexes of several bacterial cells (Fig. 2E–H). *Actinomyces* appeared as irregularly shaped long rods (Fig. 3A–D), occasionally forming intertwining filamentous structures (Fig. 3E, F).

4. Discussion

In summary, the presence and levels of *Synergistetes* was evaluated in apical samples of teeth diagnosed with N-AP or R-AP. As clinical control, a pulpitis group was employed, whereas as microbiological controls, the presence of well-established endodontic pathogens (i.e. *Fusobacteria* and *Actinomyces*) was evaluated. Sampling from the root canal system is not free from methodological pitfalls [21]. One problem with root canal sampling, as with periodontal sampling, is the fact that microorganisms from sites other than the front of the lesion are collected along with counterparts that cause disease progression. In the current study, it was aimed to sample from the apical aspect of the root canal system, and thus, NaOCl was used to lyse the bacteria that may have been present in the coronal aspects of the root canal. However, as can be seen by the fact that *Fusobacteria* could be sampled in 11% of the irreversible pulpitis cases, which should essentially be bacteria-free in the apical region [11], we cannot necessarily claim that the current sampling procedure predictably prevented false-positive results in the sense that bacteria were transported from the tooth crown to the apex during root canal preparation prior to sampling. Nevertheless, the current results clearly show that there is a vast difference in numbers of recovered taxa between teeth with apical periodontitis and counterparts with irreversible pulpitis.

Table 2
Frequency of detection of different taxa in apical samples per clinical diagnosis.

	Pulpitis	N-AP	R-AP	*P* Value
Synergistetes cluster A	0/27 (0%)	3/33 (10%)	5/21 (24%)	0.022
Synergistetes cluster B	0/27 (0%)	0/33 (0%)	0/21 (0%)	N/A
Actinomyces	0/27 (0%)	4/33 (12%)	10/21 (48%)	<0.0001
Fusobacteria	3/27 (11%)	4/33 (12%)	8/21 (38%)	0.027

Table 3
Range of detection (bacterial counts) of different taxa in the apical samples per clinical diagnosis.

	Pulpitis	N-AP	R-AP
Synergistetes cluster A	Not detected	50–90	70–255
Synergistetes cluster B	Not detected	Not detected	Not detected
Actinomyces	Not detected	70–600	55–1650
Fusobacteria	50–105	70–135	50–720

It was found that *Synergistetes* cluster A bacteria were detected in both N-AP and R-AP but not in pulpitis. Their prevalence as well as detection range was greater in R-AP compared to N-AP. *Synergistetes* cluster B was not detected in any of the three clinical diagnosis groups, implying that it is not crucial in the establishment of AP, as has also been demonstrated in the case of marginal periodontal diseases [6,8]. Earlier works used other molecular assays to detect *Synergistetes* in samples obtained from the apical part of N-AP or R-AP-affected teeth and analyzed by 16S rRNA-based nested or hemi-nested PCR, or by reverse-capture checkerboard hybridization assays. In these studies, the main *Synergistetes* oral clones detected included BA121 (now designated as *Pyramidobacter piscolens*), BH017/D084, W090 (now designated as *Fretibacterium fastidiosum*) and E3_33 (now designated as *Jonquetella anthropi*), and were found at prevalence of up to 33% [16,17,22,23]. Among those, *Synergistes* oral clone BA121 (*P. piscolens*) was the most frequently detected (29%) [23,24], at levels exceeding 10^5 DNA counts [25], and was also among the most frequently detected taxa in endodontic abscesses [26]. Hence, the prevalence reported in these earlier studies using PCR-based methods is well in agreement findings of the present study for the prevalence of *Synergistetes* cluster A in N-AP and R-AP (10% and 24%, respectively). Yet, some of the previously identified species (*P. piscolens* and *J. anthropi*) belong to *Synergistetes* cluster B, while the present study failed to detect bacteria in this cluster. The possible explanation for this discrepancy is that the cluster B species described to date are smaller in size than cluster A taxa, so they may be harder to detect by FISH. Moreover, the 16S rRNA probe used in the present study was designed for a broad range of cluster B clones, and not exclusively for these two species [8]. This study additionally demonstrates the morphology of *Synergistetes* cluster A in apical samples, and confirms the absence of any *Synergistetes* in pulpitis. The structure *of Synergistetes* cluster A described here by FISH, resembles that of *Synergistetes* identified in dental plaque of gingivitis patients, using the same method [8].

Bacteria of the genus *Actinomyces* are highly prevalent in the polymicrobial communities of infected dental root canals [27], and account for the majority of Gram-positive rods identified in AP [28]. Their persistence in the root canal is associated with failed endodontic treatment and incomplete periapical healing, as identified by bacterial cultivation [29], or by PCR-based methods [30]. The high prevalence of *Actinomyces* and *Fusobacteria* in the apical region of teeth with endodontic infection has also been demonstrated by pyrosequencing analysis, which revealed great diversity in the bacterial communities of the affected teeth, as well as a great inter-individual diversity [31]. Accordingly, when the bacterial communities of the apical portion of N-AP or R-AP-affected teeth were compared, a high prevalence of *Fusobacteria* and *Actinomyces* was confirmed, with R-AP displaying a greater diversity in bacterial composition [32]. Peri-radicular lesion samples analyzed by a 454-sequencing platform revealed that among the most abundant genera, as represented by the total yield of sequences, were *Fusobacterium* and *Actinomyces* [33]. In the present study, *Actinomyces*

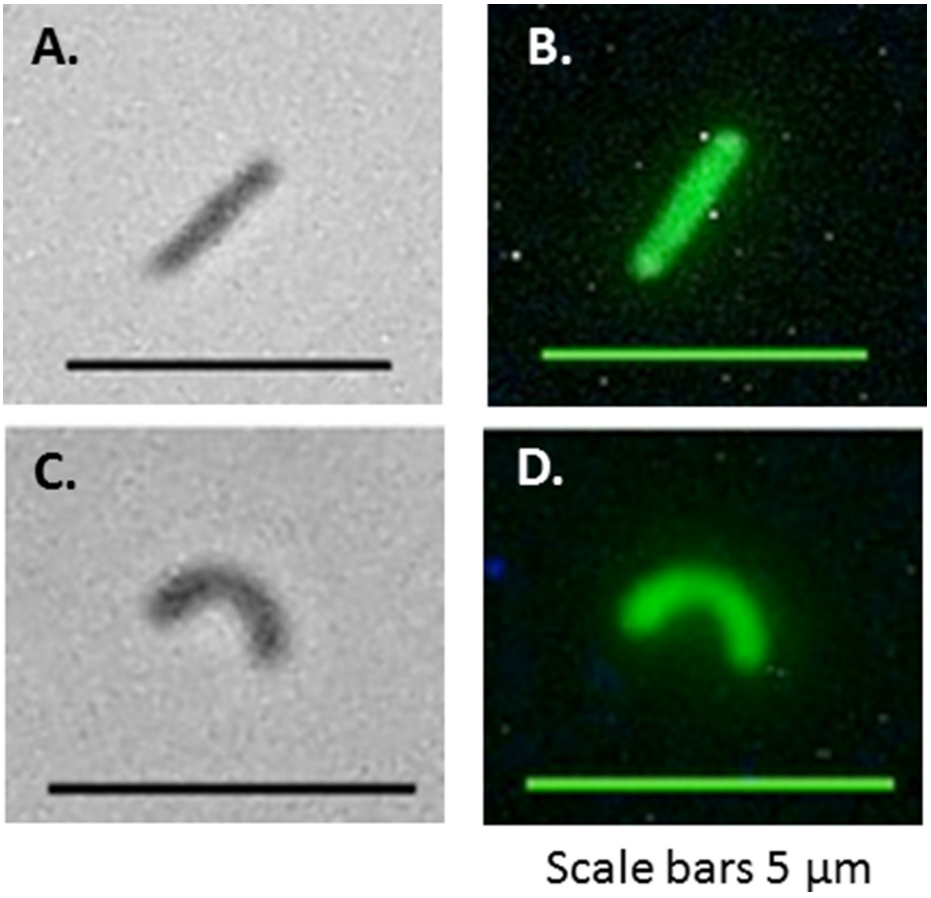

Fig. 1. Morphology of bacteria from *Synergistetes* cluster A in apical samples, as identified by FISH. (A and C) Phase contrast and (B and D) corresponding epifluorescence images stained with SYN-A1409-FAM probe. Scale bar = 5 µm.

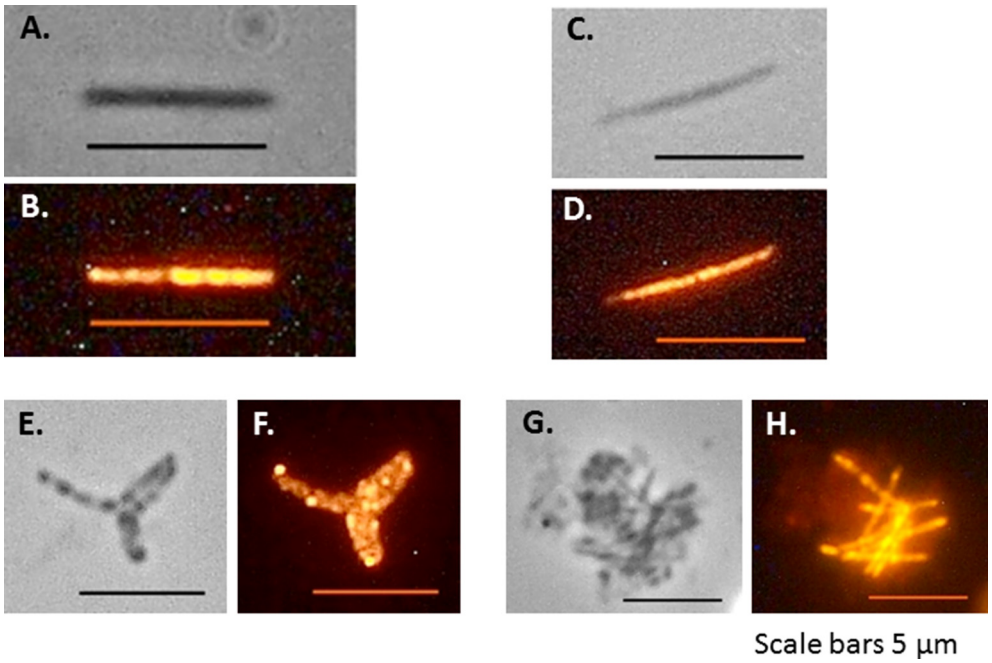

Fig. 2. Morphology of *Fusobacteria* in apical samples, as identified by FISH. (A, C, E, and G) Phase contrast and (B, D, F and H) corresponding epifluorescence images stained with FUS664-Cy3 probe. Scale bar = 5 µm.

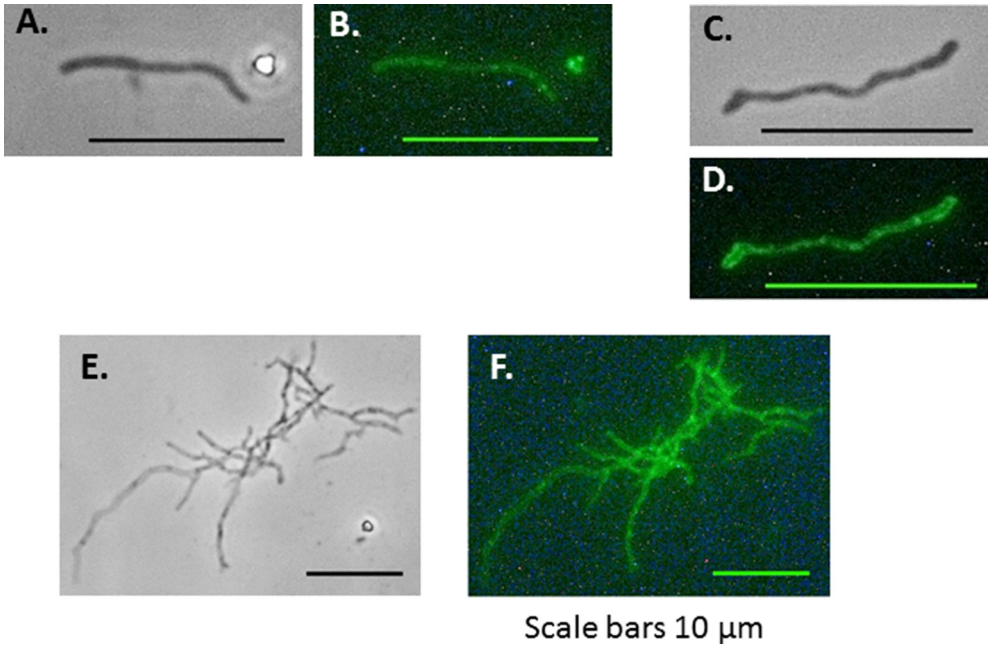

Fig. 3. Morphology of *Actinomyces* in apical samples, as identified by FISH. (A, C and E) Phase contrast and (B, D and F) corresponding epifluorescence images stained with L-ACT476-2-FAM probe. Scale bar = 10 µm.

and *Fusobacteria* were detected in both N-AP and R-AP, albeit at higher prevalence and numeric range in the latter case. These findings are well in line with the literature. The novelty of the present study is the confirmation of the presence of *Actinomyces* and *Fusobacteria* in periapical pathoses by FISH staining and microscopic visualization. With the help of the selected probes used, the bacteria could be identified the genus level, but not the phylotype level. The fact that these bacteria have been microscopically identified here proves their apical presence by culture-independent methods. However, this does not necessarily imply that they would be retrievable by cultivation, as their density was relatively low. Still, the method provides the possibility for descriptive information on the morphology of the microorganisms under investigation. Indirect immunofluorescence microscopy has been used earlier to determine the presence of selected *Actinomyces* species in endodontic infections. These were identified in 60% of the cases [34], which is proximal to the 48% prevalence in R-AP, reported in this study.

In conclusion, the findings of this study represent a snapshot of the microbial complexity in the apical root canal region. The FISH method employed here allows for the visual identification and relative enumeration of the targeted taxa, irrespective of their cultivability. The physical presence of *Synergistetes* cluster A bacteria was confirmed in both N-AP and R-AP, whereas this was absent in pulpitis. A stronger presence of this cluster was evident in R-AP, while cluster B was non-detectable in any of clinical diagnosis groups. In conclusion, *Synergistetes* cluster A, but not cluster B, bacteria can be found intact as part of the mixed apical microbiota of AP infections, and may be involved in their pathogenesis. Whether this association represents a causal relationship with the disease, or is merely a result of opportunistic infection remains to be elucidated.

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This review article that summarizes the shortcomings of current pulpal and periapical diagnostic regimes, and envisages their potential improvement is presented as an introduction to the topic:

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Molecular diagnostics in endodontics

DAN-KRISTER RECHENBERG & MATTHIAS ZEHNDER

Recent systematic reviews have substantiated the fact that current testing methods to assess the inflammatory state of the pulp and the periapical tissues are of limited value. Consequently, it may be time to search for alternative routes in endodontic diagnostics. Molecular assessment methods could be the future. However, in the field of endodontics, the research in that direction is only about to evolve. Because pulpal and periradicular diseases are related to opportunistic infections, diagnostics can either target microorganisms or the host reaction. This communication discusses clinical situations in which a molecular assessment of infection or host reaction could be of interest. The methods, collection sites, current state of research, and unresolved problems are evaluated.

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Introduction

The term "diagnosis" stems from the ancient Greek concept of relating a disease to a tangible cause rather than to the will of a higher power. Translated into English, diagnosis, from δια and γνώση, means "thorough understanding," or understanding the cause–effect relationship in a specific disease. In clinical endodontics, the cause of disease is opportunistic infection of dental hard tissues by oral or transient microorganisms (1,2). If these infections are removed, the dental pulp and periapical tissues have a high propensity to heal (3,4). Consequently, endodontic diagnostics should focus on either the extent of the microbial infection or the inflammatory reaction of the host tissue. Strangely enough, current methods do neither (5,6). Instead, pulp tests are based on triggering a nerve response, while periapical diagnostics rely on radiographic imaging. These methods have their clear benefits in everyday clinical practice, but also their limitations, which have been discussed elsewhere (5–7) and shall not be detailed in the current communication. Methods concentrating on pulpal blood flow are also beyond the scope of this communication and not discussed. Laser Doppler flowmetry (8), pulp oxyometry (9), and dual-wavelength spectrophotometry (10) can be useful in coronally intact front teeth after trauma (6). However, the application of those methods is limited by their inability to monitor teeth with restorations and lateral teeth. Therefore it is unlikely that they will gain much relevance in restorative dentistry and endodontics.

The great majority of papers on pulp diagnosis have focused on the question: is the pulp histologically or clinically vital or necrotic? Based on this dichotomized outcome, the sensitivity and specificity of a test can be calculated. In clinical practice, however, other questions are asked, such as: "will the pulp survive after I place a permanent restoration?" or "will the periapical inflammation recede after root canal treatment has been initiated?" These questions are not addressed at all with current diagnostic tools.

This paper will focus on the possibility of diagnosing pulpal and periapical inflammation via molecular markers. As is discussed in the following text, this approach could reveal information from a spatially restricted area, which may help to save vital tissues.

A brief take on the status quo of endodontic diagnostics

Monitoring pulpal health

The history of a tooth can have a great impact on the treatment decision. However, in the context of correct diagnosis of the state of the pulp, this information can only contribute to, but not tell, the overall picture. It has been shown that whether or not a tooth has a history of pain does not necessarily reveal much about its condition (11,12). Monitoring pulpal health is therefore recommended during routine dental visits and after operative procedures aimed at maintaining pulpal vitality. The most common method is the cold test, using either a carbon dioxide pencil or a cotton pellet saturated with refrigerant spray (1,1,1,2-tetrafluoroethane). The application of cold to tooth crowns stimulates vital, thermo-sensitive nerve fibers. In the case of a sensitive pulp, the patient perceives pain. Further methods are the application of heat, electric currents (electric pulp test), or even test drilling through present restorations. However, the validity of such tests is frequently limited in teeth with coronal restorations, with pulp canal obliteration, or after trauma. The advantages and disadvantages of the respective methods are discussed in detail elsewhere (5–7,13). Nevertheless, it is worth mentioning that all of the common pulp tests solely rely on the patient's subjective response to a stimulus. Depending on several factors such as anxiety, cultural background, level of analgesia by painkillers, or the patient's mood, this stimulus can be perceived differently. Because current pulp tests are not able to identify the state of the pulp, they should be called "sensitivity tests" rather than "vitality tests."

Monitoring periapical health

Teeth with a negative sensitivity test are checked radiographically to identify the presence or absence of apical periodontitis. The presence of a periapical radiolucency is correlated with pulpal necrosis and histological signs of inflammation at the periapex (14). However, intact tissues including nerve fibers have been observed histologically in the pulp stump of the apical root third, despite the presence of an apparent periapical lesion (15). Moreover, on periapical radiographs, it can take up to 1 year to see a decisive change in the appearance of the periapex (16). A further problem with two-dimensional imaging is anatomical blurring (17,18). Apical periodontitis in cancellous bone, for example, can be non-detectable radiographically, even though extensive disease is present (17,18). In theory, three-dimensional imaging may overcome this problem (19). However, radiation to oral tissues with current methods is still not acceptable for standard monitoring (20). Furthermore, while such data exist for conventional radiographs (14,21), the correlation between the histology of periapical tissues and their radiographic appearance has not been established in three-dimensional images. As an example, it is not known what scar healing would look like on cone-beam images (22). Moreover, as shown most recently, healthy teeth can have a widened ligament in cone-beam images (23).

Pulpal and periapical inflammation

In the context of diagnostics, it would appear to be of utmost importance to understand the inflammatory diseases that we treat or try to prevent in everyday clinical practice. Our focus should especially be on the location of the inflammation for a given condition (Fig. 1).

Pulpal inflammation, in the vast majority of cases, is triggered by microorganisms. These enter the pulp space either through caries (15), traumatic fractures or cracks in the dentin (24), or, in the absence of a functioning pulp, exposed dentinal tubules (25). The oral microbiota interacts with the hard tissues of the tooth crown. This is also why the infectious process initiates in the crown or the cervical area of the tooth. As the infection progresses from the coronal to the apical aspects of the root canal system, a radiographic lesion around the portals of exit of the root canal system becomes apparent. The radiographic lesion reflects the advancing periapical bone resorption, which is part of the host response to the presence of microorganisms in the root canal (26). As the disease process continues, biofilm formation occurs all the way into the complex anatomy of the root apex (27), which can be extremely difficult to debride and disinfect (28,29). This may be seen as the biological reason why the presence or absence of a periapical lesion prior to intervention is the main predictor for the radiological outcome of root canal treatment (30,31).

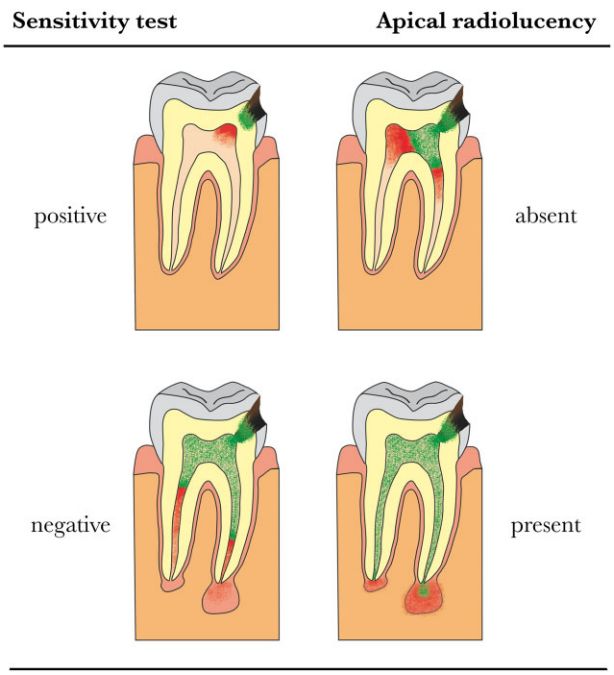

Fig. 1. Schematic depiction of different histiological states of the pulp and the periapex that can be encountered with similar results obtained by current diagnostic tests. Bacterial invasion is represented in green; inflammatory infiltrate in red. With a positive pulp test, in the absence of radiolucency, the pulp may already be infected and thus irreversibly inflamed, at least in its coronal aspect (top right). However, with a negative pulp test and an apical radiolucency, parts of the pulp tissue may still be vital and not necessarily infiltrated with inflammatory cells (bottom left). This diagram should highlight the need for better, site-specific diagnostics that can reveal the state of the pulp and/or the periapex.

From the aspect of preventive dentistry, it should be clear that hindering the progression of pulpal infection should be the aim. Conceptually, as Paracelsus knew 500 years ago: "If you prevent infection, Nature will heal the wound by herself" (32). At the same time, vital, non-infected tissues should be preserved. *Primum non nocere* (Scribonius Largus, approximately 50 AD) has remained a core ethical principle in medicine (33). This is currently not done, or at least not consequently practiced in everyday clinics. As will be discussed below, the lack of proper diagnostic methods plays a major role in this context. Or in other words: as long as we do not know what we treat, overtreatment seems to be easier and more predictable than preservation of vital tissues. As an example, if we attempt to maintain vitality in a pulp, which is, in reality, irreversibly inflamed, the patient may experience excruciating pain in the future (Fig. 1, top right). In a retrospective study on the survival of pulps after direct pulp capping procedures in teeth with a normal response to the cold test, a mere 20% of the pulps remained vital after 10 years (34). This also suggests that at least 50% of the teeth that lost pulpal vitality during the observation period should have caused some form of memorable discomfort to the patient (12).

Treatment concepts: from the crown to the apex and back again

Maintaining coronal pulp vitality

Infection of the dentin–pulp continuum usually initiates in the coronal aspect of the tooth and progresses apically. The main reason is caries, which will be discussed in some detail. However, cracks in the dentin should not be left unmentioned, as they can be the hidden cause of irreversible pulpitis and its typical clinical symptoms (35). Regardless of the route of microbial challenge to the pulp, it is not quite clear when an actual intervention into the pulp is necessary and when it is not. In caries that does not reach into the inner ⅓ of the dentin, complete excavation is not mandatory. A tight coronal seal is apparently sufficient to arrest the process (36). The same may be true for caries that reaches into the inner ⅓ on the preoperative radiograph. At least in the short term, stepwise excavation has consistently performed better than full excavation in terms of maintaining pulp vitality (37–39). However, patients experiencing spontaneous pain prior to treatment were excluded from these studies. If pain is taken as an outcome, there was no difference between stepwise excavation and direct pulp capping. In the most detailed and scientifically sound randomized trial on the topic of complete versus partial excavation to date (39), 8 of the 143 patients in the stepwise excavation group experienced unbearable pain leading to pulpectomy within the first year after treatment, compared to 7 of the 149 patients in the complete excavation group ($P = 0.93$). Long-term follow-up studies exist only for direct pulp capping. These show a consistent yearly failure rate, culminating in maintained pulpal vitality as low as 20% after 10 years (34).

Another, often overlooked, reason for losing pulpal vitality is devitalization through the preparation of teeth for indirect fixed reconstructions. Systematic reviews have suggested that roughly 10% of abutment teeth pulps become necrotic within 10 years after restoration (40). However, the true number may be higher, as these reviews included studies that did not specifically focus on the assessment of pulp vitality. Cheung and co-workers followed 199 initially vital abutment teeth for a mean of 15 years (41). Of these teeth, 77 were bridge abutments while 122 were restored with single metal-ceramic crowns. Of the bridge abutments, 32.5% became necrotic during the observation period, compared to 15.6% of the crowned teeth ($P < 0.05$). This is in line with the classic study on teeth with advanced periodontal disease that were included in long-span bridges (42). Of the abutment teeth, 15% developed pulpal necrosis over 9 years, compared to 3% of the teeth that were not included in the reconstructions.

Pulpotomy versus pulpectomy

In current clinical practice, the treatment of teeth with necrotic, partially vital, and vital pulps is the same: full pulpectomy. The designated treatment goal of pulpectomy is the removal of all organic substrate from the root canal system (43). However, this is not based on any biological or clinical fact (44) but rather on radiological esthetics. Earlier authors realized that dentin chips obtained from instrumenting non-infected root canals are the perfect biomaterial to seal off root canals (45,46). The preparation goal in many schools was to instrument 3 mm short of the apical foramen in vital cases, to maintain a vital pulp stump in the apical root canal system (47). But this concept has not stood the test of time. The reason for instrumenting to full working length is probably two-fold: the endodontist can show his or her skills on the radiograph, which can then also be appreciated at recall by another dentist, who may not know whether a tooth contained a vital pulp prior to treatment or had apical periodontitis. The second reason is that during endodontic treatment it is currently not possible to know whether the remaining tissue is free of bacterial infection or not. Conversely, it may be so that in most vital teeth with irreversible pulpitis, it is unnecessary to instrument and fill the root at all. Complete pulpotomy, i.e. the removal of the coronal pulp without entering the root canals, in combination with a tight coronal seal, could become a valid treatment in cases of irreversible pulpits (48). Some randomized trials have pointed in that direction (49,50). Unfortunately, the authors of these articles submitted the same data to different journals simultaneously. Their articles on pulpotomy versus pulpectomy were removed from the *Journal of Dental Research* and the *Journal of Endodontics*. Consequently, we have to wait for results from other groups to validate these potentially important data.

Pulp tissue engineering

Tissue regeneration strategies have gained substantial attention in the dental literature in recent years (51). Amongst the regenerative dental procedures, revascularization of a necrotic pulp space appears to be the treatment option that holds the most promise for the immediate future (52). The idea of revascularizing a necrotic pulp space in order to induce continued root formation is not new. In fact, it is a common procedure in dental traumatology (53). The first report to describe this procedure in teeth affected by caries and its sequelae dates back half a century (54). However, a subsequent study from that time in vital and non-vital human teeth of adult patients showed that revascularization was only possible in teeth that had contained a vital pulp prior to treatment (55). The tooth revascularization concept disappeared for three decades, only to resurge some years ago in the form of case reports (56). The so-called revascularization procedure is deemed to be especially helpful in children with teeth with incomplete root formation. In the first visit, the root canal system is disinfected by irrigation with a sodium hypochlorite solution and the placement of an antibiotic paste. In a following visit, the paste needs to be removed, and after inducing bleeding from the apical tissues, the blood clot in the coronal aspect of the root canal is sealed off using a calcium-silicate cement covered by a permanent restorative material (57). Ideally this procedure results in continued root development in length and width. However, recent observations question whether new dentin formation really occurs. What probably happens is that, once the infection is treated, periodontal tissues grow into the pulp space of the immature root with the open apex (58). Recent advances in tissue engineering and cell homing could

make it possible to extend this concept to adult teeth that contain some vital pulp tissue (59).

Why could molecular diagnostics be helpful?

It would be good if a patient's subjective response to traditional pulp sensitivity tests could be overcome. Furthermore, diagnostic methods should be more site-specific and accurate than the tests we currently have. This would enable the avoidance of overtreatment and the maintenance of vital tissues. Such methods could help to delineate the level of instrumentation necessary in a given clinical situation. In restorative dentistry, this could help to determine whether a pulp requires any kind of treatment prior to restoration or not. This would be important for direct or indirect pulp capping procedures as well as for the assessment of the general pulpal health before the replacement of fillings or indirect restorations. In terms of the root canal treatment itself, it would be good to know whether a pulp can be kept in its entirety, or, if pulpotomy or amputation of the pulp is intended, at which level this procedure should be executed. Furthermore, if pulpectomy is performed in teeth with apical periodontitis, it would be helpful to know whether the inflammation in the periapex is about to heal prior to obturation of the root canal system.

For all of the above tasks, molecular analysis of the site of intervention could be of diagnostic value. As we deal with opportunistic infections, the first question that arises in this context is whether it would be preferable to identify and/or analyze molecules that reflect bacterial presence or the inflammatory state of the involved tissues.

Bacteria or host response?

Targeting bacteria allows for a direct assessment of the cause of disease. Historically, the root canal content was cultured either to confirm the reduction or absence of bacteria prior to obturation, or to define the individual bacterial composition of the infection. The latter was done to generate an antibiogram on which the clinician was supposed to choose the suitable antibiotic for the therapy (60). However, endodontic infections have been shown to be non-specific and composed of mixed microorganisms (61). They are driven by ecological factors rather than specificities of the invading species, and thus the concept of the "ecological plaque hypothesis" (62) should not only be extended to periodontal but also to endodontic infections (63). Taken together, those circumstances make it rather unlikely that it might be possible to identify an individual key microorganism, or even a specific group of microorganisms, of superior diagnostic value. The elusiveness of site-specific microorganisms, which are routinely present at the forefront of endodontic infections but absent from other oral sites, renders root canal sampling for diagnostic purposes error-prone. Culture methods, once mandatory for dental students, have been questioned and abandoned for routine root canal treatments (44). In addition, there are methodological pitfalls associated with culturing microorganisms from root canals (64). Contamination is always a problem. Meticulous precautions need to be applied in order to avoid false positive results when microbial samples are collected from root canals for research purposes (65,66).

As a more straight-forward alternative to culturing, it may be possible to identify sessile bacterial aggregates directly by simple optical methods (Fig. 2). It has been shown that bacterial colonies can be excited with filtered xenon light (405 ± 20 nm) and viewed through a 530-nm high-pass filter (67). This approach is used in cariology for conservative caries excavation (68). There is now a commercially available system on the market called FACE (fluorescence-aided caries excavation, Sirona, Bensheim, Germany). Effectively, FACE does not trace bacteria, but rather porphyrine compounds in dentin associated with microbial presence (69). Theoretically, this method could be useful in vital pulp therapy. After a pulpotomy procedure, it could be used to assess whether the remaining tissue or the root canal dentin is infected or not. However, it is currently unknown whether fluorescence methods are suitable for tracking pulpal infection. Studies should be performed to determine this.

In the context of molecular diagnostics, there are some clear advantages to assessing the host response rather than bacterial invasion. That is probably why there have been more approaches in that direction. Each site and tissue fluid has its specific environment of host factors (70–72). This fact, *per se*, can

Fig. 2. Longitudinal section of a molar affected by caries excited with violet light (370–420 nm) and observed through a 530-nm high-pass filter (A). This method is applied clinically in a system known as FACE (fluorescence-aided caries excavation). A clinical picture (B) shows a premolar before complete caries excavation. The method highlights porphyrin rings, which are abundant in the presence of certain bacteria. This can be appreciated by directly monitoring bacterial colonies: *Prevotella intermedia* growing on blood agar depicted using the FACE method (C). These images are courtesy of Prof. Wolfgang Buchalla, Regensburg, Germany.

circumvent the problem of contamination with e.g saliva. The composition of the respective analyte is altered during an inflammatory process. Therefore factors associated with disease can be associated or controlled against counterparts associated with health.

Which fluid to collect?

Depending on the given clinical situation, the intervention may aim to maintain pulpal vitality or regain periapical health. Logic would dictate that different clinical situations need to be approached with different tests. Biologically, the main target should be to locate the bacterial invasion in the continuum of the pulp space and the periapex (Fig. 1). The following text will discuss sampling fluids and sites.

Gingival crevicular fluid (GCF)

GCF is an exudate that derives from the gingival crevice. It contains a multitude of host factors including antibodies, bacterial antigens, proteins, and cytokines (73). In the late 1950s the idea was born to correlate molecular markers of GCF with periodontal inflammation, because GCF is produced and modified by the periodontal ligament (74). Since then many studies have shown that host factors are differentially present in the GCF of periodontally affected teeth compared to healthy counterparts (75). The same has been shown for orthodontically induced root resorption (76) and trauma-induced root resorption (77). All of those observations share the feature that periodontal inflammation is assessed, regardless of the underlying cause. This is possible because the neural and vascular supplies of the periodontium and endodontium are functionally and anatomically connected (78,79). Since collecting GCF is a non-invasive procedure, it might thus provide diagnostic information for any given clinical situation. However, studies investigating changes of host factors in GCF of endontically involved teeth are relatively sparse. Some studies showed that the composition of neurotransmitters, interleukin-8, or MMP-8 differs in GCF from painful versus non-painful teeth (80–83). Other investigations evaluated the differences in the molecular composition of GCF between teeth with asymptomatic apical periodontitis compared to non-diseased controls (84–86).

GCF analyses are deemed to be promising by some researchers because apical periodontitis, similar to marginal periodontitis, is a local inflammation of the periodontal ligament. Indeed, in theory it might even be possible to assess the dynamics of apical periodontitis (e.g. healing of apical periodontitis after root canal treatment) using GCF. However, one major drawback in the evaluation of host mediators in GCF is that tissue inflammation, independent of its cause, is a non-specific process of the innate immunity (87). This renders it difficult, if not impossible, to distinguish on a molecular level between a marginal and an apical periodontal inflammation. Thus, there will always be bias from gingival or periodontal inflammation in GCF analyses targeted at pulpal or periapical disease. Furthermore, the GCF is unlikely to be able to reflect the spatial level of microbial progress in the pulp space, which, as delineated above, should dictate treatment decisions. The gingival crevice is thus probably not the most ideal collection site for molecular diagnostics in endodontics.

Pulpal blood

Blood and its cellular components play an essential role in the specific and the non-specific immune system. Hemogram analyses are a standard of care in general medical practice and enable us to draw conclusions on a variety of conditions with an effect on blood composition. Blood from the pulp can carry factors that differ from those in the peripheral blood (88), indicating that there is site-specific information to be gained from this fluid.

The first person to investigate a pulpal blood hemogram was Dr. Florian Prader at the University of Zürich (70). In 1949 he compared pulpal blood smears of teeth with advancing endodontic infection to their inflammatory status assessed by histology and concluded that progressing bacterial involvement is associated with a quantitative elevation of cellular blood compounds, most prominently polymorphonuclear neutrophil granulocytes (PMNs). About fifteen years later, Guthrie and co-workers published their classic study on pulpal blood examination (89). They compared clinical signs and symptoms of endodontically involved teeth with pulpal blood smears and the histological status of those same teeth after extraction. The authors made two main observations, which are worthy of being repeated word-for-word:

(i) "The fact that a high percentage of the pulps in the poor risk group bled profusely from the exposed site upon removal of the caries, gave limited support to the belief that excessive hemorrhage from an exposed pulp is one contraindication for vital pulp therapy."

(ii) "It was interesting to note that none of the teeth which exhibited high neutrophil counts were found to be in the good risk group (histologically)."

This study highlighted again the pivotal role of PMNs in the breakdown of the pulp.

Since the Guthrie study, only two studies have used modern molecular techniques (enzyme-linked immunosorbent assay, ELISA) to evaluate human pulpal blood with respect to molecular markers at the protein level (90,91). Nakanishi and co-workers looked at upstream markers in the inflammatory cascade, and found that IgG, IgA, IgM, elastase, and especially prostaglandin E2, but not IL-1 or IL-6, were elevated in inflamed compared to healthy teeth. A recent study found that the neutrophil-attracting chemokine IL-8 was significantly elevated in symptomatic pulpitis teeth compared to clinically healthy counterparts and teeth with mere caries exposure of the pulp (91).

One reason that this field of research did not gain much attention in the past may be that sampling pulpal blood requires entering the pulp space. It has been shown that entering the pulp space decreases the chance that the pulp will survive (39). Nevertheless, current scientific inventions aimed at pulpal revascularization or regeneration have in common that the pulp space is accessed. In such situations, molecular diagnosis of pulpal blood might provide valuable information on the condition of the pulp. There may be an impasse though, and that is the fact that blood interferes with most protein assays (92,93).

Dentinal fluid

Dentinal fluid is the extracellular fluid that is contained within dentinal tubules (94). The idea to sample dentinal fluid from the dentin wound to assess the state of the pulp stems from the research group of Professor Pashley (95). It was suggested to assess factors associated with PMNs, which makes a lot of sense, as was already noted by Guthrie and co-workers (89), and will be discussed later in this text. The Pashley group performed a multitude of studies on fluid flow through dentin in dog teeth. The first study on protein levels in dentinal fluid in human teeth collected *in situ* was performed by Knutsson and co-workers (96). These authors filled buccal cavities with saline solution and collected the content of the cavity 15 min after application to analyze serum albumin levels. This is clinically not practical and highlights the main problem with dentinal fluid collection: low yields of protein. In *in vitro* studies this problem may be overcome by collecting the entire fluid content of an extracted tooth by occlusal trimming and centrifugation (97). However, this is not possible in studies on vital teeth, and thus samples have been pooled from multiple teeth (98). This approach, for obvious reasons, cannot be taken in diagnostics.

In a relatively recent study on dentinal fluid analysis in clinically healthy and highly painful teeth affected by the clinical signs of irreversible pulpitis, folded polyvinylidene difluoride (PVDF) membranes were

used to collect dentinal fluid from exposed dentin. The dentin wound was dried by pressured air prior to collection (99). MMP-9 was identified by a highly sensitive assay in 7 of the 16 teeth diagnosed with irreversible pulpitis, compared to zero MMP-9-positive samples from the 12 healthy control teeth. The inconsistent recovery of MMP-9 in the clearly symptomatic pulpitis teeth was related to low yields and the possible inability of the PVDF membrane to absorb the fluid from the tubules. As has been shown in the context of GCF collection and analysis, the type of vehicle that is used has a great impact on protein absorption and release (100). Consequently, the authors performed a study to compare different vehicles for possible dentinal fluid collection *in vitro* and *in vivo* (101). It was found that large-pore cellulose membranes yielded more of a molecule of diagnostic value (MMP-2) compared to PVDF counterparts. However, more research is necessary to assess whether or not dentinal fluid analysis could ever become clinically feasible and meaningful.

It is certainly true that, if procedural problems can be resolved, dentinal fluid diagnostics would be most helpful in all of the dental interventions that aim to maintain a healthy coronal pulp. Dentinal fluid analysis could shine some light on the clinically unresolved questions of whether or not a pulp has a chance to survive indirect pulp capping or the mere preparation of the tooth as an abutment for an indirect restoration.

Periapical fluid

The term "periapical fluid" or "periapical tissue fluid" refers to the extracellular fluid present in the periapical area. If the periapical tissues become inflamed, the periapical fluid becomes an exudate and its composition is modified (102). There are different sub-types of exudates. In the case of severe inflammation, for example, an exudate can become purulent and contains dead cell fragments (e.g. from PMNs). Because exudate formation is an immediate host reaction, analysis of the exudate may provide useful information about the current state of inflammation. Collecting periapical fluid during root canal treatment is feasible after full pulpectomy. Ascending periapical fluid has been collected either by inserting a paper point or a methylcellulose strip to working length, or by aspiration with a syringe (72,103,104). Studies on the apical root anatomy of permanent human teeth have shown that the diameter of the apical constriction is usually ≥ ISO size 20 (105). Therefore, it is even possible to protrude a small-sized paper point slightly beyond the apical constriction to collect the tissue fluid present there (106).

One outstanding feature of analyzing periapical fluid is that it enables researchers to study the inflammatory response to a microbial infection in a closed environment in humans (107). The background noise of mediators not induced by the immediate disease process, a common problem with GCF analysis, is not present. Bacteria-related factors such as endotoxin and host-related factors such as cytokines, immunoglobulins, and MMPs have been identified from periapical exudates in human root canals (72,108–110). The concentrations or ratios of some factors in periapical fluid have been shown to correlate with clinical signs and symptoms (111–113), the presence of purulent exudate (102,114), or the size of the preoperative apical radiolucency (104,108,115). Furthermore, a relationship between root canal treatment and the decrease of certain factors has been established (102–104,110). Overall, those studies suggest that periapical fluid can be analyzed for molecular markers and that their concentration can change under different clinical conditions.

Periapical fluid could be especially helpful in monitoring the early signs of healing in a two-visit treatment approach (110,114,116,117). In contrast to the slow recession of periapical radiolucencies, the change in molecular composition of periapical fluid appears to occur quickly, and thus a chairside assay could be helpful in deciding at the beginning of the second endodontic visit whether a root canal system can be filled or requires more thorough disinfection.

Which target molecules should be considered?

Pulpitis and apical periodontitis are caused by mixed opportunistic infections. The host response to these is collectively termed the innate or non-specific immune system. Histological studies clearly show that pulpitis is a PMN-driven inflammation (15,118). In fact, the PMNs form a wall of defense against the microbial invaders (26). PMNs produce an entire array of proteolytic enzymes (119,120). These digest soft tissue and make it possible for the PMNs to infiltrate

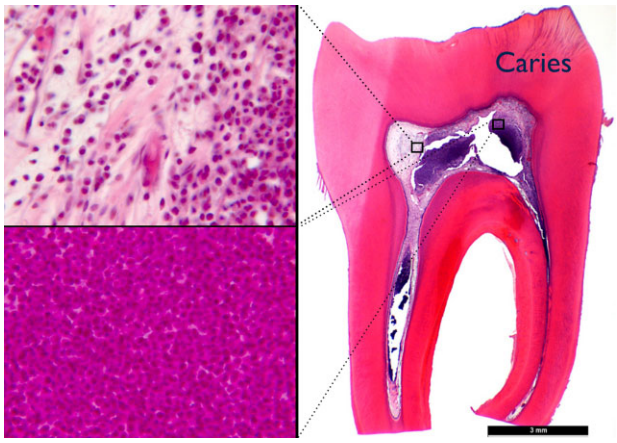

Fig. 3. Microscopic view of a histological specimen from a mandibular molar affected by caries. Note the heavy infiltration of the pulp tissue with polymorphonuclear neutrophil granulocytes (PMNs), leading to abscess formation and pulp necrosis. Courtesy of Prof. Emeritus Hans-Ueli Luder, University of Zürich.

the site of bacterial invasion. However, the innate defense mechanism also has its limits. If the microbial invaders are not removed from the tooth, abscess formation ensues (121) and the pulp tissue necrotizes. In the context of disease progression, this means that the host gradually loses its access to the site of microbial presence (Fig. 3). In the periapical tissues, the situation is slightly more complex because bone resorption occurs concomitantly to the recruitment of the inflammatory infiltrate (107). Nevertheless, the basics of the immune response remain similar in the pulp space and the periapical area.

Studies on entire pulp tissue consistently showed that PMN-related enzymes such as elastase and cathepsin G (122,123) and MMP-9 (124) are elevated in clinically inflamed pulps compared to healthy dental pulps. Histologically, the presence of these enzymes or their respective mRNAs is strongly correlated to the inflammatory infiltrate (125,126). The same is true for IL-8, the main neutrophil chemoattractant. At the gene expression level, IL-8 levels are distinctly different between clinically healthy and inflamed human dental pulps, while upstream genes encoding for early inflammatory responses such as IL-1β do not differ between these clinical states (127). IL-8 expression is also spatially correlated with the inflammatory infiltrate (128).

When it comes to monitoring periapical health, the picture is less clear (107). This is probably because some studies aimed at identifying factors associated with apical periodontitis investigated chronic lesions undergoing apical surgery rather than progressive (acute) lesions (129). These lesions could effectively represent relatively healthy tissue with little propensity to ever become acute (130).

Based on these biological givens, it makes most sense to monitor PMN-related factors in molecular diagnostics. Less site-specific, upstream inflammatory markers such as IL-1β are not suitable as markers for pulpitis (90,127), and probably also not for apical periodontitis. Most interestingly, in all of the studies that have been performed thus far comparing clinically healthy and diseased teeth by assessing PMN-related markers in GCF, pulpal blood, dentinal fluid, or periapical fluid, a significant difference was found between the two states (Table 1). PMN factors could thus also be key in identifying an ongoing inflammatory process in the periapex (110). A recent study by the authors showed the absence of IL-8 at the protein level in the periapical fluid of teeth with irreversible pulpitis, in contrast to counterparts with apical periodontitis (131). Alternatively, factors associated with bone resorption could be targeted, but these appear to provide less unequivocal results (132).

As clinicians, perhaps unknowingly, we already take PMN activity into account when treating teeth with apical periodontitis and a fistula. If the fistula disappears after the first visit, we know that healing has initiated. If the fistula is still there at the second visit, we know that we need to improve our root canal disinfection protocol and/or consider the rarer possibility of a self-sustaining extraradicular infection (133).

Chairside assays: will they ever be possible?

In dentistry, few sophisticated diagnostic tests have ever made it to the market. The main reason for this is probably that the cost and time for these assays do not justify their application. Nevertheless, it is interesting to note that in periodontology, molecular testing methods have found their way into clinics (134), even though potential overtreatment by the dental hygienist is cheaper than most of the tests that are currently on the market, with little or no side-effects for the patient. In endodontology, all of the procedures are usually performed by a specialized

Table 1: Studies on PMN markers to diagnose the inflammatory status of the pulp or the periapex*

Reference	Substrate	Target molecule(s)	Significant result**
Karapanou et al. 2008 (82)	GCF	IL-8	yes
Shin et al. 2011 (83)	GCF	MMP-8	yes
Belmar et al. 2008 (84)	GCF	MMP-9	yes
Nakanishi et al. 1995 (90)	Pulpal blood	Elastase, prostaglandin E2	yes
Elsalhy et al. 2013 (91)	Pulpal blood	IL-8	yes
Zehnder et al. 2011 (99)	Dentinal fluid	MMP-9	yes
Wahlgren et al. 2002 (110)	Periapical fluid	MMP-8	yes
Guo et al. 2000 (111)	Periapical fluid	IL-8	yes
Shimauchi et al. 2001 (112)	Periapical fluid	IL-8	yes
Alptekin et al. 2005 (113)	Periapical fluid	Elastase	yes
Alptekin et al. 2005 (114)	Periapical fluid	Elastase, prostaglandin E2	yes

Numbers in parentheses relate to the numbers in the reference list.
Abbrevations: PMN: polymorphonuclear neutrophil granulocyte; GCF: gingival crevicular fluid.
*Only studies on individual human teeth of different clinical states are included (no pooled samples).
**Significant difference at the ≤ 5% level between clinically/histologically healthy and diseased teeth.

dentist and are therefore expensive. Furthermore, poor treatment decisions may cause severe pain to the patient. Consequently, it would make sense to develop better diagnostic tests not only from a health perspective but also from a socio-economic point of view. Ideally, these tests should be performed chairside because it makes little sense to recall a patient just because we have to wait for a test result from the lab.

The host factors discussed in this communication can, in theory, be assessed at three levels: gene expression (mRNA), protein, and active protein. Assays targeting the gene expression level are unlikely to ever be applicable in a chairside set-up. The one GCF assay that is on the market to measure soft tissue breakdown in periodontal pockets targets active MMP-8 and thus PMN activity (135,136). This assay is based on the immunochromatography principle, which uses two monoclonal antibodies specific for different epitopes of MMP-8. The assay is essentially similar to a common pregnancy test, just with a different target molecule. It has been sold under various brand names such as PerioSafe and Periomarker by different companies such as Dentognostics, Hager und Werken GmbH, and GlaxoSmithKline Consumer Healthcare. The priority date for the underlying patent (WO1996007103 A1), filed by Professor Timo Sorsa, was August 26, 1994. The patent will thus expire this year. It would be most interesting to evaluate whether such assays could also be developed for the analysis of dentinal and periapical fluid. With the former, there will most likely be a sensitivity problem as the target proteins collected from dentinal fluid are in the low ng range (101). However, with periapical fluid, yields are similar to GCF and assays could easily be developed.

Concluding remarks

In summary, we can state that much research is necessary to advance molecular diagnostics in endodontology. However, it would appear that it is worth the effort. This communication was composed to summarize information that is not normally presented in this clinical context. Apparently, few researchers have embraced this topic, but hopefully that will change in the near future. Based on the current state of knowledge, some key points should be investigated:

- detection of bacterial aggregates in the root canal/dental pulp by direct optical methods;
- improvement of protein yield in dentinal fluid collection;

- interference of pulpal blood with the assessment of target proteins; and
- correlation between PMN markers in dentinal and/or periapical fluid and clinical outcomes, such as maintained pulp vitality or healing of periapical lesions.

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